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Names approved by The Nordic Pharmacopoeia Council[1]) (NFN Names).

(Received February 21, 1962)

The Nordic Pharmacopoeia Council announces its approval of NFN[3]) names for the substances in the following list. These names may be used should the substances be included in the coming Nordic Pharmacopoeia, in one or more of the Nordic national pharmacopoeias and official formularies or in addenda to them.

The substances for which these NFN names have been approved are identified by giving first the chemical names of the substances, secondly the most frequently used nonproprietary international names, including the proposed or recommended International Non Proprietary Names (denoted pINN and rINN) approved by WHO and thirdly a selection of the best known registered trade names and other names of pharmaceutical specialities (preceded by the letter ℞) applying either to the substance itself or to salts of it or to preparations in which it is the active principle. This information is given in the same manner as in the Council's publication 'NFN navne'[4]), both the volume of information and the use of abbreviations.

BAN British Approved Name
NND New and Nonofficial Drugs

Names approved in 1961[2])

NFN name	*Other Names*
Acidum benzamidosalicylicum calcium salt: calcii benzamidosalicylas (BAN rINN)	4 Benzamido-2 hydroxybenzoic acid ℞ Aminacyl B PAS, Bentsopas, Benzacyl, Benzapas, Benzido-pas, Bepas, Therapas NND Benzoylpas *(Chemotherapeutic)*

[1]) The General Secretary Frederikssundsvej 378 Copenhagen, Brønshøj Denmark
[2]) Names approved in 1960 see this journal vol 18 218–30, 1961
[3]) NFN Abbreviation of the Nordic name ("Nordisk Farmakopé navn") for the Nordic Pharmacopoeia Council
[4]) NFN navne 2nd edition Copenhagen Helsingfors, Reykjavik, Oslo and Stockholm 1958 with Addendum 1961

NFN-name	*Other Names*
Acidum docetrizoicum propyl ester propyli docetrizoas (BAN,rINN)	3-Diacetylamino-2,4,6-triiodobenzoic acid (X-ray contrast medium)
Acidum embonicum salt with dibromopropamidine dibrompropamidini embonas (BVC)	4,4'-Methylenebis(3-hydroxynaphthalenecarboxylic acid-(2)) NND Pamoic acid. (Anion in salts of certain drugs)
Acidum hexacyclonicum sodium salt natrii hexacyclonas (rINN)	2-(1-(Hydroxymethyl)cyclohexyl-(1))acetic acid (Psycho-analeptic)
Acidum jopodicum calcium salt: calcii jopodas sodium salt natrii jopodas (pINN)	3-(3-Dimethylaminomethyleneamino-2,4,6-triiodophenyl)propionic acid. pINN Natrii iopodas BAN,NND Sodium Ipodate ® Biloptin, Oragrafin sodium, Solu-biloptin (X-ray contrast medium)
Acidum theoclicum salt with promethazine promethazini theoclas (BAN,rINN)	8-Chloro-1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydrogenpurine 8-Chlorotheophylline (Anion in salts of certain drugs)
Acidum thyropropicum (rINN)	3-(4-(4-Hydroxy-3-iodophenoxy)-3,5-diiodophenyl)-propionic acid (Against hypercholesterolaemia)
Acidum xenazoicum (pINN)	4-(1-(Biphenylyl-(4)-carbonyl)-1-ethoxymethylamino)-benzoic acid ® Xenovis (Chemotherapeutic)
Allyloestrenolum (BAN,rINN)	17α-Allyl-17β-hydroxyestrene-(4) rINN Allylestrenolum ® Gestanin, Gestanon (Progestogen)
Aminoglutethimidum (rINN)	3 (4-Aminophenyl)-3-ethyl-2,6-dioxopiperidine 2-(4-Aminophenyl)-2-ethylglutarimide ® Elipten (Sedative)
Amitriptylinum chloride amitriptylini chloridum (BAN,pINN)	5-(3-Dimethylaminopropylidine)-10,11-dihydrogen-5H-dibenzo[a,d]cycloheptene ® Elavil, Saroten, Tryptizol (Psycho-analeptic)
Amphotalidum (pINN)	1-(4-Aminophenoxy)-5-phthalimidopentane (Chemotherapeutic)
Amphotericinum (BAN,rINN,NND)	Antibiotic produced by Streptomyces nodosus rINN Amphotericinum B (BAN,NND) ® Fungizone
Anisindionum (BAN,rINN)	2-(4-Methoxyphenyl)-1,3-dioxoindane ® Miradon (Anticoagulant)

NFN name	*Other Names*
Aspartocinum	Antibiotic produced by Streptomyces griseus var
(pINN)	spiralis
Bamethanum	2 Butylamino 1 (4-hydroxyphenyl)ethanol
sulfate bamethani sulfas	® Vasculat, Vasculit
(BAN pINN)	(Vasodilator)
Bendroflumethiazidum	3 Benzyl 7 sulfamoyl-6-trifluoromethyl 3,4-dihydrogen
(pINN NND)	1,2 4-benzothiadiazinedioxide (1,1)
	BAN Bendrofluazide
	® Aprinox Centyl, Naturetin Neo naclex, Salures
	(Diuretic)
Benziodaronum	2 Ethyl 3 (4 hydroxy 3 5-diiodobenzoyl)benzofurane
(pINN)	2 Ethyl 3 (4 hydroxy 3 5-diiodobenzoyl)cumarone
	® Amplivix
	(Spasmolytic)
Benzphetaminum	N Benzyl N methyl N (1 methyl 2 phenylethyl) amine
chloride benzphetamini	® Didrex
chloridum	(Anorexigenic, sympathomimetic)
(BAN pINN)	
Bephenum	N Benzyl N N dimethyl N (2 phenoxyethyl)
3 hydroxy 2 naphthoate	ammonium hydroxide
bepheni oxinaphthoas	pINN Bephenii hydroxynaphthoas (BAN)
salt of 4 4 methylenebis	BVC Bephenium Embonate
(3 hydroxynaphthalenecarbo	® Alcopar
xylic acid (2)) bepheni	(Anthelmintic)
embonas	
(BVC pINN)	
Biperidenum	1 (Bicyclo[2 2 1]heptene (5)-yl (2))-1 phenyl 3 piperi
chloride biperideni chloridum	dinopropanol (1)
lactate biperideni lactas	® Akineton
(BAN pINN NND)	(Antiparkinson agent)
Bunamijodylum	3 (3 Butyramido 2 4 6-triiodophenyl)-2-ethylacrylic acid
sodium salt bunamiyodyl	rINN Bunamiodylum (NND)
natrium	® Orabilex
(rINN NND)	(X ray contrast medium)
Butadiazamidum	2 Butyl 5 (4-chlorobenzenesulfonamido)-1,3,4
(rINN)	thiadiazole
	(Antidiabetic)
Butylhydroxitoluenum	2 6-Di *tert* butyl-4-methylphenol BHT antioxidant
	BAN Butylated Hydroxytoluene
	® Impruvol Jonol Vianol
	(Pharmaceutical necessity)
Butylscopolaminum	Hydroxide of 8 butyl 3-tropoyloxy-6,7-epoxytropane
bromide butylscopolamini	N Butylscopolamine N Butylhyoscine
bromidum	® Bulamin, Buscopan
	(Parasympatholytic)
Carbenazidum	2 (1 Phenylethyl)hydrazincarboxyl c acid (1)ethyl ester
(pINN)	(Psycho-analeptic, monoaminoxydase inhibitor)

NFN name	*Other Names*
Carbodimidcalcium	Calciumcyanamide rINN Calcii carbimidum ® Dipsan Temposil (Against alcoholism)
Carisoprodatum	2 Carbamoyloxymethyl 2 isopropylcarbamo yloxymethylpentane rINN Carisoprodolum (BAN NND) ® Carisol Rela Soma Somadril (Skeletal muscle relaxant)
Carperidinum (pINN)	1 (2 Carbamoylethyl) 4 phenylpiperidinecarboxylic acid (4)ethyl ester (Antitussive)
Chinethazonum (pINN)	7 Chloro 2 ethyl 4 oxo 6 sulfamoyl 1,2 3 4 tetrahydro genquinazoline *pINN Quinethazonum* (Diuretic)
Chlorphenoxaminum chloride chlorphenoxamini chloridum (BAN pINN NND)	2 (1 (4 Chlorophenyl) 1 phenylethoxy) N N dimethylethylamine ® Clorevan Phenoxene Systral (Antihistaminic)
Chlorphenterminum chloride chlorphentermini chloridum (BAN pINN)	2 (4 Chlorophenyl) 1 1 dimethylethylamine ® Avicol Lucofen (Anorexigenic)
Chlorserpidinum (pINN)	10 Chloro 2 methoxy 1 methoxycarbonyl 3 (3 4 5 trimethoxybenzoyloxy) 1 2 3 4 4a 5 7 8 13 13b 14 14a dodecahydrogen[g]indolo[2 3 a]quinolizine 10 Chloro 11 demethoxyreserpin pINN Chloroserpidinum (Psycho sedative)
Chymotrypsinum (rINN)	Enzyme obtained from pancreas by extraction of its proenzyme chymotrypsinogen and conversion with trypsin to chymotrypsin α Chymotrypsin ® Alpha chymar Chymar Chymolase Kymo trypure Zolyse
Cinnamaverinum *(DCF rINN)*	2 3 Diphenylacrylic acid (2 diethylaminoethyl) ester *(Spasmolytic)*
Cinnarizinum (BAN pINN)	*trans* 1 Cinnamyl 4 diphenylmethylpiperazine ® Glanil Mitronal (Antihistaminic)
Clomidazolum chloride clomidazoli chloridum	1 (4 Chlorobenzyl) 2 methylbenzimidazole pINN Chlormidazolum (BAN) ® Futrican (Fungicide)
Clonitazinum	2 (4 Chlorobenzyl) 1 (2 diethylaminoethyl) 5 nitro benzimidazole *pINN Clonitazenum (BAN)* (Analgesic euforising)

NFN name	*Other Names*
Clopoxidum chloride clopoxidi chloridum	7 Chloro 2 methylamino 5 phenyl 3H 1 4 benzodiazepineoxide (4) pINN Chlordiazepoxidum (BAN) ® Librium (Psycho sedative)
Clopyroxinum	3 (4 Chlorodiphenylmethoxy)-1 methylpyrrolidine pINN Pyroxaminum (Antihistaminic)
Crotoniazidum (pINN)	2 (Buten (2) ylidene) 1 (pyridyl (4)-carbonyl)hydrazine 2 (Buten (2) ylidene) 1 isonicotinoylhydrazine (Chemotherapeutic)
Cyclophosphamidum (BAN rINN)	2 (Bis(2-chloroethyl)amino)-1 oxa 3 aza 2 phosphacyclohexaneoxide (2) ® Cytoxan Endoxan Sendoxan (Nucleotoxic agent)
Cyproheptadinum chloride cyproheptadini chloridum (BAN rINN)	5 (1 Methylpiperidylidene (4)) 5H dibenzo[a d]cycloheptene ® Periactin (Enteramine antagonist)
Dichlorisonum acetic acid ester dichlorisoni acetas (rINN)	9α 11β Dichloro 17α 21 dihydroxy 3 20-dioxopregnadiene (1 4) ® Diloderm Disoderm (Antirheumatic steroid)
Dieldrinum (BAN pINN)	Product containing about 85 per cent 3 4 5 6 9 9 hexachloro 1a 2 2a 3 6 6a 7 7a octahydrogen 2 7 3 6 dimethanooxireno[b]naphthalene (Insecticide)
Dimeflinum (pINN)	8 Dimethylaminomethyl 7 methoxy 3 methyl 4-oxo 2 phenylchromane (Analeptic)
Dimeprozinum	9 (3 Dimethylaminopropylidene)-2 methoxyxanthene rINN Dimeprozanum (Psycho sedative)
Dimetholizinum phosphate dimetholizini phosphas (rINN)	1 (2 Methoxyphenyl)-4 (3 methoxypropyl)piperazine (Antihypertensive)
Dipiproverinum chloride dipiproverini chloridum (DCF pINN)	2 Phenyl 2 piperidino acetic acid (2 piperidinoethyl) ester ® Levospasme (Spasmolytic)
Diisoprominum (pINN)	N Diisopropyl 3 3 diphenylpropylamine pINN Diisoprominum (Spasmolytic)
Ditophalum (BAN pINN)	1 3 Ethylthiocarbonylbenzene ® Etisul (Chemotherapeutic)

NFN-name	*Other Names*
Emylcamatum (BAN,rINN)	3-Carbamoyloxy-3-methylpentane 1-Ethyl-1-methyl-propanol-carbamic acid ester. ®: Nuncital (Skeletal muscle relaxant)
Ethambutolum (pINN)	N,N′-Bis (1-hydroxymethylpropyl)ethylenediamine (Chemotherapeutic)
Ethenzamidum (pINN)	2-Ethoxybenzamide ®: Lucamid (Analgesic)
Ethionamidum (BAN,DCF,rINN,NND)	2-Ethyl-4-thiocarbamoylpyridine 2-Ethylpyridine-carbothionamide-(4) ®· Trecator, Trescatyl (Chemotherapeutic)
Ethosuximidum (BAN,pINN)	3-Ethyl-3-methyl-2,5-dioxopyrrolidine. 2-Ethyl-2-methylsuccinimide. ® Emeside, Suxinutin, Zarontin (Antiepileptic)
Etonitazinum	1-(2-Diethylaminoethyl)-2-(4-ethoxybenzyl)-5-nitro-benzimidazole pINN· Etonitazenum (BAN) (Analgesic, euforising)
Ferricholinatum	Chelate of ferric hydroxide and (2-hydroxyethyl)trimethyl ammonium dihydrogen citrate Chelate of ferric hydroxide and choline dihydrogen citrate rINN Ferrocholinatum (NND) ® Chel-iron, Ferrolip, Komplifer (Treatment of iron deficiency)
Flubenisolonum	9α-Fluoro-11β,17α,21-trihydroxy-16β-methyl-3,20-dioxopregnadiene-(1,4) 9α-Fluoro-16β-methylprednisolone pINN Betamethasonum (BAN) ® Betnelan, Celeston (Glucocorticoid)
Fluocinoloni acetonidum (BAN,pINN)	6α, 9α-Difluoro-11β,21-dihydroxy-3,20 dioxo 16α, 17α isopropylidenedioxypregnadiene-(1,4) 6α,9α-Difluoro-16α-hydroxyprednisolone acetonide-(16,17) ® Synalar (Glucocorticoid)
Fluphenazinum chloride fluphenazini chloridum (BAN,rINN,NND)	10 [3-(4-(2-Hydroxyethyl)piperazinyl-(1))-propyl]-2-trifluoromethylphenothiazine trifluoromethylphenothiazine ® Moditen, Pacinol, Permitil, Prolixin (Psycho-sedative)
Glucagonum chloride· glucagoni chloridum (pINN)	Pancreatic hormone, polypeptide consisting of 29 amino acids with a minimum molecular weight of 3482

NFN name	*Other Names*
Guaneth dinum sulfate guanethidine sulfas hydrogen sulfate guanethidini b sulfas (BAN pINN)	(2 Octahydrogenazocinyl (1)-ethyl)guanidine ® Ismelin (Sympatholytic)
Haloperidolum (BAN pINN)	4 (4 Chlorophenyl) 1 (3 (4 fluorobenzoyl)propyl-4 hydroxypiperidine ® Haldol Serenase (Psycho sedative)
Haloponum chloride haloponi chloridum	N (4 Bromobenzyl) 3 (4-chloro 5 methyl 2 isopropyl phenoxy)propyldimethyl ammonium hydroxide rINN Halopenii chloridum (BAN) (Antibacterial)
Haloprogesteronum (pINN)	17α Bromo 6α fluoro-3 20 dioxopregnene-(4) 17α Bromo 6α fluoroprogesterone (Progestogen
Hexapropymatum (BAN rINN)	1 *Carbamoyloxy 1 (propyn (2)-yl)cyclohexane* 1 (Propyn (2) yl)cyclohexanol-carbamic acid ester ® Lunamin Merinax Modirax (Sedative)
Hexcarbacholinum bromide hexcarbacholini bromidum (rINN)	N N Hexamethylenebis[(2 carbamoyloxyethyl)trimethyl ammonium hydroxide] (Skeletal muscle relaxant)
Homochlorcycli inum (rINN)	1 (4 Chlorodiphenylmethyl)-4 methyl 2 3 4 5 6 7 hexa hydrogen 1 4 diazepine (Enteramine antagonist)
Hydroflumethiazidum (BAN rINN)	7 Sulfamoyl-6-trifluoromethyl 3,4 dihydrogen 1,2 4 benzothiazinedioxide (1 1) ® Hydrenox Naclex Rontyl Saluron Vergonil (Diuretic)
Hydromorphinolum (BAN pINN)	3 6 14 Trihydroxy 17 methyl-4 5-epoxymorphinane 14 Hydroxy 7 8-dihydrogenmorphine (Analgesic euforising)
Hydroxocobem num	α (5 6-Dimethylbenzimidazolyl)hydroxocobamide pINN Hydroxocobalaminum (Vitamin (B group) haematopo etic)
Ibylcainum form ate ibylcaini formias chloride ibylcaini chloridum	4 Aminobenzoic acid (2 isobutylaminoethyl) ester NF Butethamine Hydrochloride ® Monocaine (Local anaesthet c)
Im nophenimidum (pINN)	3 Ethyl 2 6 dioxo-3 phenylpiperazine (Hypnotic)
Isoaminilum citrate isoamin li citras (BAN pINN)	3 Cyano-5-dimethylamino-3 phenyl 2 methylhexane 4-D methylamino-2 phenyl 2 isopropylvaleronitril ® Dimyril Peracon (Antitussive)

NFN-name	*Other Names*
Emylcamatum (BAN,rINN)	3-Carbamoyloxy-3-methylpentane 1-Ethyl-1-methyl-propanol-carbamic acid ester. *®: Nuncital* (Skeletal muscle relaxant)
Ethambutolum (pINN)	N,N'-Bis (1-hydroxymethylpropyl)ethylenediamine (Chemotherapeutic)
Ethenzamidum (pINN)	2-Ethoxybenzamide *®: Lucamid* (Analgesic)
Ethionamidum *(BAN,DCF,rINN,NND)*	2-Ethyl-4-thiocarbamoylpyridine 2-Ethylpyridine-carbothionamide-(4) ® Trecator, Trescatyl (Chemotherapeutic)
Ethosuximidum (BAN,pINN)	3-Ethyl-3-methyl-2,5-dioxopyrrolidine. 2-Ethyl-2-methylsuccinimide. ® Emeside, Suxinutin, Zarontin (Antiepileptic)
Etonitazinum	*1-(2-Diethylaminoethyl)-2-(4-ethoxybenzyl)-5-nitro-benzimidazole.* pINN: Etonitazenum (BAN) (Analgesic, euforising)
Ferricholinatum	Chelate of ferric hydroxide and (2-hydroxyethyl)trimethyl ammonium dihydrogen citrate Chelate of ferric hydroxide and choline dihydrogen citrate rINN Ferrocholinatum (NND) ® Chel-iron, Ferrolip, Komplifer (Treatment of iron deficiency)
Flubenisolonum	9α-Fluoro-11β,17α,21-trihydroxy-16β-methyl-3,20-dioxopregnadiene-(1,4) 9α-Fluoro-16β-methylprednisolone pINN. Betamethasonum (BAN) ® Betnelan, Celeston (Glucocorticoid)
Fluocinoloni acetonidum (BAN,pINN)	6α, 9α-Difluoro-11β,21-dihydroxy-3,20-dioxo-16α, 17α-isopropylidenedioxypregnadiene-(1,4) 6α,9α-Difluoro-16α-hydroxyprednisolone acetonide-(16,17) ® Synalar (Glucocorticoid)
Fluphenazinum chloride fluphenazini chloridum (BAN,rINN,NND)	10-[3 (4-(2-Hydroxyethyl)piperazinyl-(1))-propyl]-2-trifluoromethylphenothiazine *trifluoromethylphenothiazine* ® Moditen, Pacinol, Permitil, Prolixin (Psycho-sedative)
Glucagonum chloride glucagoni chloridum (pINN)	*Pancreatic hormone, polypeptide* consisting of 29 amino acids with a minimum molecular weight of 3482

NFN name	*Other Names*
Guanethidinum sulfate guanethidine sulfas hydrogen sulfate guanethidini bisulfas (BAN pINN)	(2 Octahydrogenazocinyl (1)-ethyl)guanidine ® Ismelin (Sympatholytic)
Haloperidolum (BAN pINN)	4 (4 Chlorophenyl) 1-(3 (4 fluorobenzoyl)propyl-4 hydroxypiperidine ® Haldol Serenase (Psycho sedative)
Haloponum chloride haloponi chloridum	N (4-Bromobenzyl)-3 (4 chloro 5 methyl 2 isopropyl phenoxypropyldimethyl ammonium hydroxide rINN Halopenii chloridum (BAN) (Antibacterial)
Haloprogesteronum (pINN)	17α Bromo 6α fluoro 3 20-dioxopregnene (4) 17α Bromo-6α fluoroprogesterone (Progestogen
Hexapropymatum (BAN rINN)	1 Carbamoyloxy 1 (propyn (2) yl)cyclohexane 1 (Propyn (2) yl)cyclohexanol-carbamic acid ester ® Lunamin Merinax Modirax (Sedative)
Hexcarbacholinum bromide hexcarbacholini bromidum (rINN)	N N Hexamethylenebis[(2-carbamoyloxyethyl)trimethyl ammonium hydroxide] (Skeletal muscle relaxant)
Homochlorcyclizinum (rINN)	1 (4 Chlorodiphenylmethyl)-4 methyl 2,3 4 5 6 7 hexa hydrogen 1 4 diazepine (Enteramine antagonist)
Hydroflumethiazidum (BAN rINN)	7 Sulfamoyl 6 trifluoromethyl 3 4 dihydrogen 1 2 4 benzothiazinedioxide (1 1) ® Hydrenox Naclex Rontyl Saluron, Vergonil (Diuretic)
Hydromorphinolum (BAN pINN)	3 6 14 Trihydroxy 17 methyl-4,5-epoxymorphinane 14 Hydroxy 7 8 dihydrogenmorphine (Analgesic euforising)
Hydroxocobeminum	α (5 6 Dimethylbenzimidazolyl)hydroxocobamide pINN Hydroxocobalaminum (Vitamin (B group) haematopoietic)
Ibylcainum formiate ibylcaini formias chloride ibylcaini chloridum	4 Aminobenzoic acid (2 isobutylaminoethyl) ester NF Butethamine Hydrochloride ® Monocaine (Local anaesthetic)
Iminophenimidum (pINN)	3 Ethyl 2 6-dioxo-3 phenylpiperazine (Hypnotic)
Isoaminilum citrate isoaminili citras (BAN pINN)	3 Cyano-5-dimethylamino-3 phenyl 2 methylhexane 4 Dimethylamino 2 phenyl 2 isopropylvaleronitril ® Dimyril Peracon (Antitussive)

NFN name	*Other Names*
Isocarboxazidum (BAN pINN)	3 ((2 Benzylhydrazino)carbonyl) 5 methyl isoxazole ® Marplan (Psycho analeptic monoamine oxydase inhibitor)
Ketohexazinum (pINN)	4 6 Dimethyl 3 oxo 2 3 dihydrogenpyridazine pINN Cetohexazinum (Hypnotic)
Levocarbinoxaminum tartrate levocarbinoxamini tartras	( ) [2 ((4 Chlorophenylpyridyl (2))methoxy)ethyl] dimethylamine ( ) Carbinoxamine pINN Rotoxamini tartras ® Twiston (Antihistaminic)
Mebhydrolinum naftalenedisulfonate (1 5) mebhydrolini naphthalendisulfonas (BAN rINN)	5 Benzyl 2 methyl 1 2 3 4 tetrahydrogenpyrido[4 3 b] indole ® Fabahistin Incidal (Antihistaminic)
Medroxiprogesteronum acetic acid ester medroxiprogesteroni acetas (pINN NND)	*17α Hydroxy 6α methyl 3 20 dioxopregnene (4) 17α Hydroxy 6α methylprogesterone* pINN Medroxyprogesteroni acetas (NND) ® Perlutex Provera (Progestogene)
Mepenzolonum bromide mepenzoloni bromidum	3 Benziloyloxy 1 1 dimethylpiperidinium hydroxide *rINN Mepenzolati bromidum (NND)* ® Cantil (Anticholinergic)
Mephenoxalonum (rINN)	5 ((2 Methoxyphenoxy)methyl) 2 oxooxazolidine (Psycho sedative)
Mepivacainum chloride mepivacaini chloridum (pINN)	*(±) 2-(2 6 Dimethylphenylcarbamoyl)* 1 methyl piperidine ® Carbocain (Local anaesthetic)
Mestanolonum (BAN rINN)	17β Hydroxy 17α methyl 3 oxo 5α androstane ® Androstalone (Androgen non virilizing)
Metahexamidum (BAN rINN)	3 (3 Amino 4 methylbenzenesulfonyl) 1 cyclohexyl carbamide ® Euglycin Melanex (Antidiabetic)
Metaxalonum (pINN)	2 Oxo 5 (3 5 xylyloxymethyl)oxazolidine (Psycho sedative skeletal muscle relaxant)
Methaqualonum chloride methaqualoni chloridum (BAN rINN)	2 Methyl 4 oxo 3 (tolyl (2)) 3 4 dihydrogenquinazoline ® Holodorm Melsedin Revonal Sovelin (Hypnotic)
Methastyridonum (pINN)	2 2 Dimethyl-4 oxo 5 styryloxazolidine (Psycho analeptic)

NFN name	*Other Names*
Methazolamidum (BAN rINN)	5 Acetylimino-4 methyl 2 sulfamoyl-4 5 dihydrogen 1 3 4 thiadiazole ® Neptazane (Carboanhydrase inhibitor)
Methdilazinum (BAN rINN NND)	10-(1 Methylpyrrolidyl (3) methyl)phenothiazine ® Dilosyn Tacaryl (Antihistaminic)
Methiomeprazinum (pINN)	(±) 10 (3 Dimethylamino 2 methylpropyl) 2 methyl thiophenothiazine (Antiemetic)
Methoserpidinum (BAN pINN)	2 10 Dimethoxy 1 methoxycarbonyl 3 (3 4 5 trimethoxybenzoyloxy) 1 2 3 4 4a 5 7 8 13,13b 14 14a dodecahydrogen[glindolo [? 3 a] quinolizine 11 Demethoxy 10-methoxyreserpin ® Decaserpyl (Hypotensive)
Methyclothiazidum (BAN pINN)	6 Chloro 3 chloromethyl 2 methyl 7 sulfamoyl 3 4 dihydrogen 1 2 4 benzothiadiazinedioxide (1,1) ® Enduron (Diuretic)
Methylaminopterinum	N [4 ((2 4 Diaminopteridyl-(6))dimethylamino) benzoyl] glutamic acid rINN Methotrexatum (BAN USP) ® Amethopterin (Nucleotoxic agent)
Methylchromonum (BAN rINN)	3 Methyl-4 oxochromene ® Crodimyl (Coronary vasodilator)
Methylprampinum nitrate methylprampini nitras	Hydroxide of 8 methyl 3 (2 phenyl 3 (propionyloxy) propionyloxy)tropane N methyl 0 propionylatropine BAN Prampine methonitrate ® Pamn (Anticholinergic)
Methysergidum hydrogen maleate methysergidi bimaleas (pINN)	4 7 Dimethyl-4 6 6a 7 8 9 hexahydrogenindolo[4 3 fg] quinolinecarboxylic acid (9)-(1-(hydroxymethyl) propylamide) N (1 (hydroxymethyl)propyl)-4 methyl D lysergamide ® Deseril (Enteramine antagonist)
Metronidazolum (BAN pINN)	1 (2 Hydroxyethyl) 2 methyl 5 nitroimidazole ® Flagyl Metronil (Against trichomonas)
Naphthononum (pINN)	2 Hydroxy 1 (2-oxocychohexyl)naphthalene 2 (2 Hydroxynaphthyl (1))cyclohexanone (Antitussive)
Natrii carbazochromsulfonas (rINN)	– (Haemostatic)

NFN-name	*Other Names*
Neallymalum	5-Allyl-2,4,6-trioxo-5-neopentylhexahydrogenpyrimidine 5-Allyl-5-neopentylbarbituric acid pINN Nealbarbitalum BAN Nealbarbitone ® Censedal, Nevental (Hypnotic)
Nialamidum (BAN,rINN,NND)	N-(N-Benzyl-2-carbamoylethyl)-N'-(pyridyl-(4) carbonyl)hydrazine N-(N-Benzyl-2-carbamoylethyl)-N'-isonicotinoylhydrazine ® Niamid (Psycho-analeptic, monoamine oxydase inhibitor)
Nicothizonum	3-Thiosemicarbazonomethylpyridine Nicotinaldehyde thiosemicarbazone rINN: Nicothiazonum (DCF) (Chemotherapeutic)
Nifuramizonum	1-(2-Dimethylaminoethyl)-1-(5-nitrofurfurylideneamino)carbamide rINN Nifurethazonum (Antibacterial)
Nifurhydrazonum	1-Acetyl-2-(5-nitrofurfurfurylidene)hydrazine rINN: Nihydrazonum. (Antibacterial)
Nifuroximum	1-Hydroxyiminomethyl-5-nitrofurane 5-Nitro-2-furaldehydeoxime. (Fungicide)
Nifurthilinum	2-*Thio*-1-(5-*nitrofurfurylideneamino*)imidazolidine pINN Thiofuradenum (*Antibacterial*)
Norvinisteronum (pINN)	17β-Hydroxy-3-oxo-17α-vinylestrene-(4) (*Progestogen*)
Octatroponum bromide octatroponi bromidum	Hydroxide of 3-(2-propylvaleryloxy) 8-methyltropane rINN Octatropini methylbromidum ® Valpin (Anticholinergic)
Oestriolum	3,16α,17β-Trihydroxyestratriene-(1,3,5(10)) Estriol, Theelol ® Destriol, Leostriol, Ovesterin, Ovestin, Tridestin, Triovex (Oestrogen)
Oximetholonum (BAN,pINN)	17β-Hydroxy-2-hydroxymethylene-17α-methyl-3-oxo-*androstane* pINN Oxymetholonum (BAN) ® Adroyd, Anadrol, Anapolon (Anabolic, androgen)
Oxindasolum acetic acid ester oxindasoli acetas	3-(2-Aminoethyl)-5-hydroxy-1-(4-methoxybenzyl)-2-methylindole. rINN Hydroxindasatum (Enteramine antagonist)

NFN name	*Other Names*
Oxiphencyclimınum chloride oxiphencyclimini chloridum (rINN,NND)	2-Cyclohexyl-2-phenylglycolic acid ((1-methyl-1,4,5,6-tetrahydrogenpyrimidyl (2))methyl) ester rINN Oxyphencycliminum (NND) ® Daricol, Daricon, Vio-thene (Anticholinergic, atropine-like)
Palmidrolum (rINN)	N (2 Hydroxyethyl)palmitamide (Antiflogistic)
Paromomycinum sulfate paromomycini sulfas (BAN rINN)	Antibiotic produced by Streptomyces rimosus and other Streptomyces species ® Humatin, Humycin, Pargonyl
Pentapiperidum hydrogen fumarate pentapiperidi bifumaras (rINN)	3 Methyl 2 phenylvaleric acid (1 methyl piperidyl-(4)) ester ® Lyspafen (Anticholinergic)
Phanchinonum (rINN)	5,6 Dioxo-5 6 dihydrogen-4,7 phenanthroline 4,7-Phenanthrolinequinone (5,6) rINN Phanquinonum BAN Phanquone ® Entobex (Amoebicide)
Phebutazinum (pINN)	1,4-Bis(2 (2 phenylbutyroyloxy)ethyl)piperazine (Spasmolytic)
Phenampromidum (BAN pINN)	1-Piperidino 2-(N-propionylanilino)propane (Analgesic)
Phencyclidinum chloride phencyclidini chloridum (BAN pINN)	1-(1 Phenylcyclohexyl)piperidine ® Sernyl (Analgesic)
Phendimetrazinum hydrogentartrate phendimetrazini bitartras (BAN pINN)	(+) 3,4 Dimethyl-2 phenylmorpholine ® Plegine (Anorexigene)
Phenethicillinum potassium salt phenethicillinkalium (BAN pINN)	4 Carboxy 5 5 dimethyl 2 (1 (2 phenoxypropionamido) carboxymethyl)thiazolidine β lactam Phenoxyethyl-penicillin ® Alfacillin Alfocillin, Bendralan, Broxil, Maxipen, α Oracillin, Pheno M penicillin, s p-Penita, Synapen, Synerpenin (Antibiotic)
Phenforminum chloride phenformini chloridum (BAN pINN,NND)	1-Phenethylbiguanide ® DBI, Dibein, Dibotin, Fenormin (Antidiabetic)
Phenglutarimidum chloride phenglutarimidi chloridum (BAN,rINN)	3 (2 Diethylaminoethyl)-2,6-dioxo-3 phenylpiperidine 2-(2 Diethylaminoethyl)-2 phenylglutarimide ® Aturban (Antiparkinson agent)

NFN-name	*Other Names*
Phemdiemolum	5,5-Diethyl-2,4,6-trioxo-1-phenylhexahydrogenpyrimidine 5,5-Diethyl-1-phenyl barbituric acid rINN. Phetharbitalum ® Pyrictal (Anticonvulsive)
Phemprazinum chloride phemprazini chloridum (BAN,pINN)	(1-Benzylethyl)hydrazine (1-Phenylpropyl (2))hydrazine ®. Catran, Catron, Cavodil (Monoamine oxydase inhibitor, psychoanaleptic, anti hypertensive)
Phenoperidinum (BAN,pINN)	1-(3-Hydroxy-3-phenylpropyl)-4-phenylpiperidinecarboxylic acid (4) ethyl ester (Analgesic, euforising)
Phenprobamatum (rINN)	1-Carbamoyloxy-3-phenylpropane 3-Phenylpropylcarbamic acid ester ® Gamaquil (Psycho-sedative, skeletal muscle relaxant)
Phenprocumonum (BAN,pINN,NND)	4-Hydroxy-2-oxo-3 (1-phenylpropyl)-2H-chromene *4-Hydroxy-3-(1-phenylpropyl) cumarine* pINN Phenprocoumonum (BAN,NND) ® Liquamar, Marcoumar *(Anticoagulant)*
Phenterminum (BAN,pINN)	1,1-Dimethyl-2-phenylethylamine ® Duromine, Ionamin (ion exchange complex) *(Anorexigen)*
Phytomenadioli natrii phosphas	Diester of 1,4-dihydroxy-2-methyl-3-phytylnaphthalene and sodium dihydrogen phosphate rINN Phytonadioli natrii diphosphas ® Kayhydrin, Mephyton DK (Vitamin (K-group))
Pipamazinum (BAN,rINN,NND)	10 (3-(4-Carbamoylpiperidino)propyl)-2-chlorophenothiazine ® Mornidine (Psycho sedative, antiemetic)
Piperacetazinum (pINN)	2-Acetyl-10-[3 (4-(2-hydroxyethyl)piperidino)propyl]-phenothiazine (Psycho sedative)
Piperylonum (pINN)	4-Ethyl-1-(1-methylpiperidyl-(4)) 5-oxo 3 phenyl-2-pyrazoline ® Palerol (mixture with tropenziloni bromidum) (Analgesic, spasmolytic)
Pipethanatum chloride pipethanati chloridum (rINN,NND)	Benzilic acid (2-piperidinoethyl) ester ® Sycotrol (Psycho-sedative)
Piprocurarum iodide piprocurari jodidum (pINN)	*Dihydroxide of 2 (1-methylpiperidinium)-2-phenyl acetic acid* [2-(2-(N,N-diethyl-N-methylammonium) ethoxy)ethyl] ester pINN Piprocurarii iodidum (Curare-like action)

NFN name	*Other Names*
Prampinum (BAN pINN)	3 (2 Phenyl 3 (propionyloxy)propionyloxy)tropane 0 Propionylatropine (Anticholinergic)
Protokylolum chloride protokyloli chloridum (rINN NND)	1 (3 4-Dihydroxyphenyl)-2 (2 (1 3 benzodioxolyl (6)) 1 methylethylamino)ethanol ® Caytin Caytina Caytine (Bronchodilating)
Pseudoephedrinum chloride pseudoephedrini chloridum (BAN pINN)	(+) 2 Methylamino 1 phenylpropanol (+) Pseudoephedrine L-(+) Ephedrine ® Sudafed (Bronchodilator sympathomimetic)
Pyrophendanum (pINN)	1 (1 Methyl 3 pyrrolidinyl (3) methyl)-3 phenylindane (Spasmolytic)
Rol tetracyclinum (pINN)	4 Dimethylamino-3 6 10 12 12a pentahydroxy 6 methyl 1 11 dioxo 1 4 4a 5 5a 6 11 12a octahydrogennaphtha cenecarboxylic acid (2) (pyrrolidinyl (1) methylamide) N (Pyrrolidinyl (1) methyl)tetracycline ® Syntetrin Syntodecin Velacycline (Antibiotic)
Sala o sulfadimid num (BAN pINN)	3 Carboxy-4 hydroxy-4 (4 dimethylpyrimidyl-(2)- sulfamoyl)azobenzene pINN Salazosulfadimidinum BAN Salazosulphadi midine ® Azudimidin (Chemotherapeutic)
Spironolactonum (BAN pINN)	Lactone of 7α acetylthio 17α (2-carboxyethyl) 17β hydroxy 3 oxoandrostene (4) 7α Acetylthio 3 oxoan drostene (4) 17 spiro 2 (5 oxotetrahydrogenfurane) ® Aldactone (Anti aldosterone)
Styramatum (BAN rINN NND)	2 Carbamoyloxy 1 phenylethanol ® Linaxar Sinaxar (Skeletal muscle relaxant)
Sulfamonomethoxinum (pINN)	4 Methoxy 6 sulfanilamidopyrimidine. (Chemotherapeutic)
Sulfaphena olum (BAN rINN)	1 Phenyl 5 sulfanilam dopyrazole BAN Sulphaphenazole ® Orisul Orisulf (Chemotherapeutic)
Sulfasomi olum (BAN rINN NND)	3 Methyl 5 sulfanilamidoisothiazole BAN Sulphasomizole (Chemotherapeutic)
Sulfatolamidum (BAN rINN)	Salt of 1 Sulfanilylthiocarbamide and 4-aminomethyl benzenesulfonamide Salt of sulfathiocarbamidum and mafenidum BAN Sulphatolamide ® Marbadal Marbadal C, Mirbedal (Chemotherapeutic)

NFN-name	*Other Names*
Tetrabenazinum (BAN,pINN)	3-Isobutyl 9,10-dimethoxy-2-oxo-1,2,3,4,6,7 hexahydrogen-11bH-benzo[a]quinolizine ® Nitoman (Psychopharmakon)
Thiamphenicolum (rINN)	D (+)-Threo 2-dichloroacetamido-1-(4 methylsulfonyl phenyl)propanediol (1,3) ® Thiocymetin (Chemotherapeutic)
Thiethylperazinum maleate thiethylperazini maleas (BAN,pINN)	2-Ethylthio-10 (3-(4-methylpiperazinyl (1))propyl)-phenothiazine ® Torecan (Antiemetic)
Thihexinolonum bromide thihexinoloni bromidum	*trans*-4 (Dithienyl (2) hydroxymethyl)cyclohexyltrimethyl ammonium hydroxide rINN Thihexinoli methylbromidum ® Entoquel (Anticholinergic)
Thioproperazinum metansulfonate thioproperazini methansulfonas (BAN,rINN)	2-Dimethylsulfamoyl-10 (3-(4-methylpiperazinyl (1))-propyl)phenothiazine ® Majeptil (Psycho sedative)
Thiotepa (BAN,rINN,NND)	Triaziridinyl (1)-phosphinsulfide N', N'', N'''-Triethylenethiophosphortriamide NND Thio tepa ® Tifosyl (Nucleotoxic agent)
Thiramum (pINN)	Bis(dimethylthiocarbamoyl)disulfide Tetramethylthiuramdisulfide (Antibacterial, fungicide)
Toloxichloralum	2 (2,3-Bis(1-hydroxy-2,2,2 trichloroethoxy)propoxy) toluene rINN Toloxychlorinolum (Hypnotic)
Tranylcyprominum sulfate tranylcypromini sulfas (BAN,pINN)	*trans* (±)-2-Phenylcyclopropylamine ® Parnate (Psychopharmakon, monoamine oxydase inhibitor)
Triclazatum (rINN)	Benzilic acid (1 methylpyrrolidinyl (3) methyl) ester *(Anticholinergic)*
Trichlormethiazidum (pINN)	6-Chloro 3-dichloromethyl 7-sulfamoyl 3,4 dihydrogen 1,2,4-benzothiadiazinedioxide-(1,1) ®. Fluitran, Flutra, Metahydrin (Diuretic)
Trichlormethinum (pINN)	Tris(2-chloroethyl)amine BAN Trimustine ® Trillekamin (Cytotoxic)

NFN name	*Other Names*
Triclobisonum chloride triclobisoni chloridum (rINN NND)	Hexamethylenebis[dimethyl(1 methyl 3 (2 2 6 trimethylcyclohexyl)propyl) ammonium hydroxide] rINN Triclobisonii chloridum ® Triburon chloride (Antibacterial)
Trifluoperazinum chloride trifluoperazini chloridum (BAN DCF,rINN NND)	10-(3 (4 Methylpiperazinyl (1))propyl 2 trifluoromethyl phenothiazine ® Stelazine Terfluzin (Psycho sedative)
Triflupromazinum chloride triflupromazini chloridum (rINN NND)	10 (3 Dimethylaminopropyl) 2 trifluoromethylphenothiazine ® Vesprin (Psycho sedative)
Trimecainum (pINN)	N (Diethylaminoacetyl) 2 4 6 trimethylaniline (Local anaesthetic)
Trimethobenzamidum chloride trimethobenzamidi chloridum (rINN NND)	N (4 (2 Dimethylaminoethoxy)benzyl 3 4 5 trimethoxybenzamide ® Tigan Hydrochloride (Antiemetic)
Trimethoprimum (pINN)	2,4-Diamino 5 (3 4 5 trimethoxybenzyl) pyrimidine (Antibacterial)
Triparanolum (BAN pINN NND)	2 (4 Chlorophenyl) 1 (4-(2 diethylaminoethoxy)phenyl)-1 tolyl (4)-ethanol ® MER/29 Trikosterol (Against hypercholesterolaemia)
Trolnitratum phosphate trolnitrati phosphas (rINN NND)	Tris(2 hydroxyethyl)aminetrinitric acid ester Triethanolaminetrinitric acid ester Aminotrate ® Angitrit Metamine Nitretamin Nitroduran Praenitrona (Vasodilator)
Tropenzilonum bromide tropenziloni bromidum	Hydroxide of 3 benziloyloxy 7 methoxy 8 methyl-tropane pINN Tropenzilini bromidum ® Palerol (mixture with piperylonum) (Spasmolytic)
Tropicamidum (pINN)	N Ethyl N (pyridyl (4)-methyl)tropic acid amide ® Mydriaticum Roche (Mydriatic)
Valoctamidum	2 Ethyl 3 methylvaleric acid amide pINN Valnoctamidum (Psycho sedative)
Xenthioratum (pINN)	4 [1-((2 Diethylaminoethylthio)carbonyl)propyl] biphenyl (Against hypercholesterolaemia)

NFN-name	*Other Names*
Tetrabenazinum (BAN,pINN)	3-Isobutyl-9,10-dimethoxy-2-oxo-1,2,3,4,6,7-hexahydrogen-11bH-benzo[a]quinolizine ®. Nitoman (Psychopharmakon)
Thiamphenicolum (rINN)	D (+) Threo 2-dichloroacetamido-1-(4-methylsulfonyl phenyl)propanediol (1,3) ® Thiocymetin (Chemotherapeutic)
Thiethylperazinum maleate thiethylperazini maleas (BAN,pINN)	2-Ethylthio-10 (3 (4-methylpiperazinyl (1))propyl) phenothiazine ® Torecan (Antiemetic)
Thihexinolonum bromide thihexinoloni bromidum	*trans*-4 (Dithienyl (2) hydroxymethyl)cyclohexyltrimethyl ammonium hydroxide rINN Thihexinoli methylbromidum ® Entoquel (Anticholinergic)
Thioproperazinum metansulfonate thioproperazini methansulfonas (BAN,rINN)	*2-Dimethylsulfamoyl-10 (3 (4-methylpiperazinyl (1))-* propyl)phenothiazine ® Majeptil (Psycho-sedative)
Thiotepa (BAN,rINN,NND)	Triaziridinyl (1) phosphinsulfide *N', N'', N''-Triethylenethiophosphortriamide* NND Thio tepa ® Tifosyl (Nucleotoxic agent)
Thiramum (pINN)	Bis(dimethylthiocarbamoyl)disulfide Tetramethyl thiuramdisulfide (Antibacterial, fungicide)
Toloxichloralum	2-(2,3-Bis(1-hydroxy-2,2,2 trichloroethoxy)propoxy)-toluene rINN Toloxychlorinolum (Hypnotic)
Tranylcyprominum sulfate tranylcypromini sulfas (BAN,pINN)	*trans* (±) 2-Phenylcyclopropylamine ® Parnate (Psychopharmakon, monoamine oxydase inhibitor)
Triclazatum (rINN)	Benzilic acid (1-methylpyrrolidinyl (3) methyl) ester (Anticholinergic)
Trichlormethiazidum (pINN)	6-Chloro-3-dichloromethyl 7-sulfamoyl 3,4 dihydrogen 1,2,4 benzothiadiazinedioxide (1,1) ® Fluitran, Flutra, Metahydrin (Diuretic)
Trichlormethinum (pINN)	Tris(2-chloroethyl)amine BAN Trimustine ® Trillekamin (Cytotoxic)

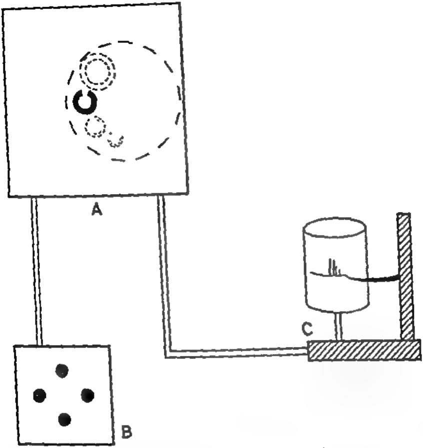

Fig 1 Diagram of the set up for testing visual acuity
A White square board) 0 5 × 0 5 m) with central Landholt ring the opening of the latter can assume any of 4 positions
B Subjects switch box with 4 contacts he presses the button corresponding to the estimated position of the Landholt ring
C Ordinate writer moves up one step when the correct button is pressed

The experimental subject is seated in a moderately illuminated room on a chair four metres distant from a white wooden box A Landholt s ring is shown in a round opening in its front wall as described below A disc A (see figure 1 and 2) with a series of transparent rings at equal intervals along the periphery is so placed inside this opening that one of the rings will appear in the opening Inside this disk there is in addition a transparent disc with an opaque sector of twenty degrees The centre of this second disc will coincide with that of the ring The transparent part of the ring forms a Landholt s ring if [illegible]

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From the Department of Pharmacology, University of Lund, Sweden

The Effect of Strychnine on Visual Acuity

By

Våge Oskarsson

(Received November 2, 1961)

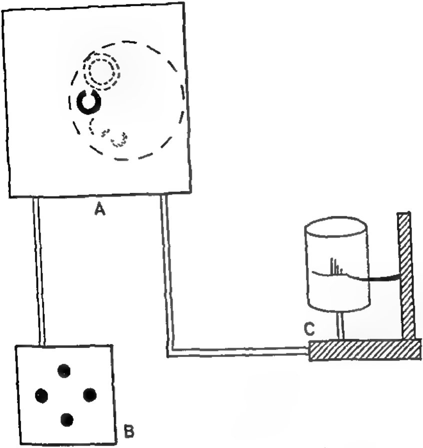
It was originally found by VON HIPPEL (1879) that vision (visual acuity, field of vision) improves after the administration of strychnine This finding lead to an extensive use of strychnine in the treatment of amblyopia and amaurosis Among later investigators WOLFFIN (1907) was able to confirm von Hippel's results, SCHLAGINTWEIT (1922) however, could not DUKE-ELDER, in Text-Book of Ophtalmology (1932), put together the available facts and stated that strychnine improves vision by facilitating synaptic transmission between ganglion cells of the retina, leading to an increased spread of the impulse generated by light stimuli

As to hearing, GISSELSSON & TOREMALM (1955) found that strychnine did not improve hearing measured by audiometry Spoken words, however, were distinguished better against a background of disturbing noise The authors concluded that the effect of strychnine on hearing is a cerebral one, the peripheral organ not being concerned

For several reasons a new investigation on the effect of strychnine on visual acuity is desirable The works done in this field have given divergent results The possible effect of placebos has not been adequately considered Finally it might be of interest to discover whether there exists any action of strychnine on visual acuity, similar to that on hearing, described above

Materials and Methods.

For the routine testing of visual acuity the letters of the alphabet are commonly used as test-types This test, however, includes higher psychic functions, e g the sense of form, besides the ability to discern light-impressions The interpretation of the patient's answers contains subjective elements To eliminate such inconveniences as far as possible the *method* described below has been used



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A White square board) 0 5 × 0 5 m) with central Landholt ring the opening of the latter can assume any of 4 positions
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The experimental subject is seated in a moderately illuminated room on a chair four metres distant from a white wooden box A Landholt s ring is shown in a round opening in its front wall as described below A disc A (see figure 1 and 2) with a series of transparent rings at equal intervals along the periphery is so placed inside this opening that one of the rings will appear in the opening Inside this disk there is in addition a transparent disc with an opaque sector of twenty degrees The centre of this second disc will coincide with that of the ring The transparent part of the ring

box with four buttons in contact with the apparatus, each of them corresponding to one of the positions of the Landholt's ring He presses the button that corresponds with the position of the ring presented as he sees it Thereby it is automatically registrated by an ordinate-writer if the answer is correct Further, a motor is set in action, on whose axis the disc B is fixed at its centre On the axis there is also an iron cross, rotating between the poles of an electromagnet When the button is released up the magnet is magnetized through a relay mechanism and so momentarily stops the motor by attracting two of the wings of the iron cross between its poles This also explains why the motor always stops with the gap in the Landholt's ring in one of four positions in a random uncontrollable way After a few seconds, demagnetization follows by the action of a condenser that permits electrical flow only for that time The subject can then try again After a number of tests on the same ring, it is automatically exchanged for a smaller one In this way a series of eight rings of decreasing diameter are presented, the later ones smaller than the earlier Simultaneously with the shift of the rings, the pen of the ordinate writer automatically falls down to the base line and moves forward one step Thus its markings indicate the number of correct answers for each Landholt's ring

To conduct an investigation, to some extent analogous to that of hearing described by GISSELSSON & TOREMALM, a special piece of apparatus has been constructed Two transparent plates of plastic having a pattern with small dark closestanding spots are placed in the projector They are made to rotate in opposite directions close together The picture is focussed on the front wall of the box, producing a disturbing varying light pattern around the Landholt's ring

Before the beginning of the experiment the subject is given time to acquaint himself with the apparatus, during which the illumination is adjusted so that he can easily see the position of the largest ring but seldom or not at all that of the smallest one In between the rings are successively smaller and as a rule the number of correct answers will fall correspondingly The subject is told to press the button at fairly regular intervals and to guess rather than try his eyes for too long a time As there are four possibilities, there is always a 25% chance of getting a correct answer by chance

Two series of experiments, each involving of 20 healthy persons between 20 and 40 years, have been carried out For each ring the subject is given 22 chances From these 22 answers it is calculated how many more or fewer correct answers are got after administration of strychnine or placebo, compared with control results, which are always determined first All figures so obtained from all the experiments are brought together and the arithmetical mean (and its mean deviation) are calculated Thus the results indicate the average improvement or impairment for each Landholt's ring after administration of the drug

Results

Series I

After determining the initial visual acuity, placebo (NaCl) was injected, and a new determination was made Then 4 mg strychnine nitrate were administrated intramuscularly and for 20–30 minutes afterwards new values for visual acuity were obtained All determinations were made both with and without the disturbing light-scatter.

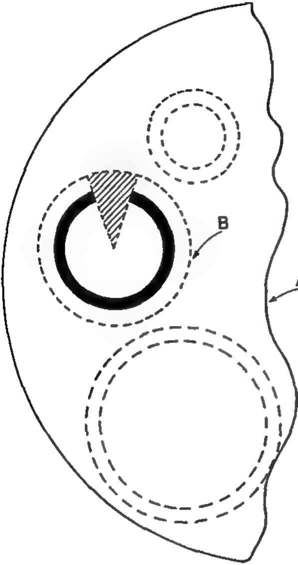

Fig 2 Details (diagramatic)

A Disc with a number of transparent rings of successively decreasing sizes After 12 trials of the subject with one ring, the disc automatically moves to the next smaller one

B Disc with an opaque wedge, seated on the axis of a motor This is started when the subject presses a button and stops at random in one of 4 positions

box with four buttons in contact with the apparatus, each of them corresponding to one of the positions of the Landholt's ring He presses the button that corresponds with the position of the ring presented as he sees it Thereby it is automatically registrated by an ordinate writer if the answer is correct Further, a motor is set in action, on whose axis the disc II is fixed at its centre On the axis there is also an iron cross, rotating between the poles of an electromagnet When the button is released up the magnet is magnetized through a relay mechanism and so momentarily stops the motor by attracting two of the wings of the iron cross between its poles This also explains why the motor always stops with the gap in the Landholt's ring in one of four positions in a random uncontrollable way After a few seconds, demagnetization follows by the action of a condenser that permits electrical flow only for that time The subject can then try again After a number of tests on the same ring, it is automatically exchanged for a smaller one In this way a series of eight rings of decreasing diameter are presented, the later ones smaller than the earlier Simultaneously with the shift of the rings, the pen of the ordinate writer automatically falls down to the base line and moves forward one step Thus its markings indicate the number of correct answers for each Landholt's ring

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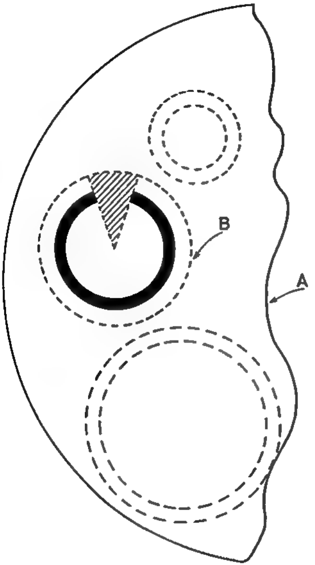

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A [illegible]

B [illegible]

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After determining the initial visual acuity, placebo (NaCl) was injected, and a new determination was made Then 4 mg strychnine nitrate were administrated intramuscularly and for 20–30 minutes afterwards new values for visual acuity were *obtained* All determinations were made both with and without the disturbing light scatter

a Without light scatter

Placebo-control $+0\ 70 \pm 0\ 34$ correct answers,

Strychnine-control $+1\ 30 \pm 0\ 30$ correct answers

Thus there are $0\ 60 \pm 0\ 45$ more correct answers after strychnine than after placebo for each ring

b With light scatter

Placebo-control $+1\ 25 \pm 0\ 32$ correct answers,

Strychnine control $+ 1\ 83 \pm 0\ 35$ correct answers,

Thus post strychnine exceed post placebo correct answers by $0\ 58 \pm 0\ 49$

The effect of strychnine appears somewhat to exceed the effect of placebo but the difference is not significand Owing to the duration of the testing (two hours) fatigue in the end might have lowered the results for strychnine On the other hand, this drawback may possibly be counterbalanced by the effect of training

Series II

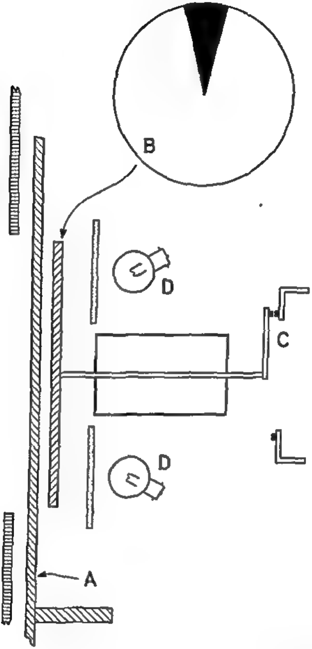
After control determination 4 mg strychnine nitrate were injected intramuscularly and visual acuity was determined 20–30 minutes later A few days later the same experiment was repeated with placebo (NaCl) in place of strychnine Every second person got strychnine on the first and placebo on the second occasion The experiment was over in 45–60 minutes so that the effect of fatigue was diminished compared with that in series I Only determinations with light scatter were carried out The results were

Placebo-control $+1\ 43 \pm 0\ 23$ correct answers,

Strychnine-control $+0\ 91 \pm 0\ 24$ correct answers

Thus a greater improvement was obtained after placebo than after strychnine (difference $0\ 52 \pm 0\ 32$ not significant) The effect of placebo is statistically significant

Conclusion The experiments have shown that a placebo *improves* visual acuity tested as above Strychnine does *not* improve the control values by more than the effect of the placebo It thus seems that under normal conditions strychnine has no other effect on visual acuity than that of a placebo, whether on the peripheral or on the central (cerebral) pathways Nor was it possible to find an effect of strychnine when the subject had to distinguish the Landholt's ring against disturbing light scatter



C 4 contacts, coupled in series with subject switchbox, enabling correct answers only to be recorded
D Electric bulbs with diffusing screen illuminating the Landholt rings from behind

Acta pharmacol et toxicol 1962, 19, 23–35.

From The Department of Pharmacology, University of Copenhagen
(Professor Knud O Møller, M D)

Morphine as an Inhibitor of Brain Cholinesterases in Morphine-Tolerant and Non-Tolerant Rats

By

Torkell Jóhannesson

(Received December 2, 1961)

BERNHEIM & BERNHEIM reported in 1936 that morphine is cholinesterase inhibiting Since then several authors, particularly SLAUGHTER and his collaborators (1940 a, b, c), have tried to explain some of the effects of morphine as being cholinergic Later work by KNOLL & KOMLÓS (1951), PORSZASZ *et al* (1951), DE JONGH (1954) and other investigators, however, did not support this assumption about the analgesic effect. Thus results have been contradictory, and the question of a close relationship between morphine (and kindred substances) and cholinergic receptors is still unsettled

Even [illegible]
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stresse [illegible]
has of [illegible]
concentration, and KRAYER *et al* (1944) and GOLDSTEIN *et al* (1949) emphasized that results from experiments with cholinesterases and reversible inhibitors are misleading if the necessary dilution of the tissue is disregarded

In our experiments with morphine and cholinesterases in rats' brains we have attempted to reduce the dissociation between enzyme and inhibitor as much as possible by correcting the dilution and by using [illegible]

[illegible] to estimate the cholinergic effect of this agent.

Summary.

The effect of strychnine on visual acuity has been investigated Landholt's rings have been used as test types in a self-recording apparatus Visual acuity sometimes improves after strychnine, but not more than after placebo, and strychnine has no significant pharmacological effect on visual acuity The experimental conditions were further complicated by introducing a disturbing light scatter around the Landholt's ring in an attempt to show any central (cerebral) effect of strychnine on vision, but there was no such effect

Acknowledgements

The author expresses his gratitude to Professor Gunnar Ahlgren, who suggested this work, and to Docent Torsten Krakau for advice on construction of the optical apparatus

The investigation was aided by a grant from the Kungliga Fysiografiska Sällskapet i Lund and from *Fil Dr P Håkanssons Stiftelse, Eslov*

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BERNHEIM & BERNHEIM reported in 1936 that morphine is cholinesterase inhibiting Since then several authors particularly SLAUGHTER and his collaborators (1940 a, b, c) have tried to explain some of the effects of morphine as being cholinergic Later work by KNOLL & KOMLÓS (1951), PÓRSZASZ *et al* (1951), DE JONGH (1954) and other investigators, however, did not support this assumption about the analgesic effect Thus results have been contradictory, and the question of a close relationship between morphine (and kindred substances) and cholinergic receptors is still unsettled

Even BERNHEIM & BERNHEIM, and later KUHN & SURLES (1938), indicated that morphine is a reversible cholinesterase inhibitor BURGEN (1949) stressed the fact that the physiological importance of reversible inhibitors has often been minimized, as the inhibition depends on the substrate concentration and KRAYER *et al* (1944) and GOLDSTEIN *et al* (1949) emphasized that results from experiments with cholinesterases and reversible inhibitors are misleading if the necessary dilution of the tissue is disregarded

In our experiments with morphine and cholinesterases in rats' brains we have attempted to reduce the dissociation between enzyme and inhibitor as much as possible by correcting the dilution and by using acetylcholine at low substrate concentrations We have thus endeavoured to obtain a reasonable manifestation of the inhibition *in vivo*, this should in some degree enable us to estimate the cholinergic effect of this agent

Summary.

The effect of strychnine on visual acuity has been investigated. Landholt's rings have been used as test types in a self-recording apparatus. Visual acuity sometimes improves after strychnine, but not more than after placebo, and strychnine has no significant pharmacological effect on visual acuity. The experimental conditions were further complicated by introducing a disturbing light-scatter around the Landholt's ring in an attempt to show any central (cerebral) effect of strychnine on vision, but there was no such effect.

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Schlagintweit, E. Über Strykninwirkung auf die Sinne, insbesondere auf das Auge. *Arch. f. exp. Path. u. Pharm.* 1922, 95, 104.

Wölfflin, E. Über die Beeinflussung der Dunkeladaption durch künstliche Mittel. *Arch. f. Opth.* 1907, 65, 302.

B *Polarographic Determination of Small Amounts of Morphine*

All determinations were performed on a polarograph. (Radiometer type Po 3h) By the method employed, described by Milthers (1958, 1959), the morphine base is transformed into morphine chloride before the polarographic determination The sensitivity of the method is 1–2 μg/g, when approximately 1 g brain tissue is used, and the average recovery is about 95%

C *Materials*

Male albino rats (120–260 g) were used After decapitation the whole brain (including the brain stem and the cerebellum) was taken out, weighed and homogenized mechanically for 1–2 minutes, with an appropriate volume of 0 9% sodium chloride solution, in a Potter & Elvehjem homogenizer By means of an ice bath the temperature was kept low before, during and after homogenization Homogenates were then diluted with 0 9% sodium chloride solution (in graduated 10 ml tubes) to give final concentrations of approximately 20–30% (w/v)

Morphine tolerance in the rats was produced by daily subcutaneous administration of morphine in progressively increasing doses The initial dose was approximately 25 mg/kg, and the final doses varied from 125 to 155 mg/kg daily, reached about 4 weeks later About 15% of the rats died during the period, most of the survivors lost [illegible]

Symbols used

[ChE] Cholinesterase activity

pS The negative logarithm of the substrate concentration

Results.

A *Morphine as an Inhibitor of Cholinesterases in Rat's Brain*

Brains from 3 non-tolerant rats were used in the experiment

The brains were homogenized together in a concentration of 20% (w/v) The total reaction volume was 25 ml at pS = 2 0 to 4 7 50 ml at pS = 5 3 and 100 ml at pS = 5 7 and 5 9 The titration fluid was 100 mM–NaOH at pS = 2 0 to 3 7 and 10 mM-NaOH at pS = 4 7 to 5 9 The substrate solution used was 250 mM, during operations at pS = 2 0 to 3 7 and 5 mM at pS = 4 7 to 5 9

The results show how changes in the substrate concentration influence morphine-produced inhibition of [ChE] in brain homogenates (fig 1) At pS = 4 0, morphine at the three concentrations employed will reduce

Methods and Materials

A Titrimetric Determination of Cholinesterase Activity

The technique used for determining cholinesterase activity in tissue homogenates is based on the method described by JENSEN HOLM LAUSEN MILTHERS & MOLLER (1959) but various later modifications from our Department have enabled us to work at substrate concentrations considerably lower than are usual (JENSEN HOLM 1960, 1961, and JÓHANNESSON & LAUSEN 1961)

Apparatus Activity measurements were made by continuous automatic titration with a titrator (*Radiometer* type TTT 1), and titrigraph (*Radiometer* type SBR 2a/SBU 1) An infusion apparatus independent of the titrator enabled us to add reagents to the reaction mixture at a constant but variable rate by which procedure any non specific acid or base liberation may be compensated (JENSEN HOLM 1962)

Reagents Where nothing else is mentioned the *titration fluid* 5mM NaOH and the *substrate* 5mM acetylcholine iodide were used The enzymatic process was begun by adding an adequate volume of this solution to the reaction mixture with a pipette *Morphine* was added in the form of a 10mM solution of the chloride (Ph Dan 1948) Boiled re distilled water was used in the experiments

Activity determinations For the determinations of cholinesterase activity we used 0 5 ml of a 20–30% homogenate of rat s brain diluted with sufficient 0 9% NaCl solution to give a total reaction mixture (after addition of inhibitor and substrate) usually amounting to 100 ml Nitrogen was bubbled through the mixture kept at 38°C and during the estimation of activity the reaction vessel being kept covered the space above the reaction mixture was swept out with nitrogen to avoid absorption of carbon dioxide The activity was determined at pH 7 40 with constant magnetic stirring Acetylcholine iodide was usually added in amounts giving a molar concentration of $6\,3 \times 10^{-6}$ (pS − 5 2) and morphine was added at concentrations corresponding to those found in rats brains after injections of morphine (see later) For practical reasons the brain tissue was incubated with morphine for 10 minutes before adding the substrate although pilot experiments showed that the equilibria between enzyme inhibitor and substrate are reached *almost instantaneously*

The basis for calculating any existing activity is the difference between the inclina- of the tangent to the titrigraphic curve one minute before and one minute after

gram tissue

Table 1

Brain cholinesterase activity [ChE] in 9 non tolerant and 5 morphine tolerant rats weighing 150–240 g The substrate was acetylcholine iod de at a molarity corresponding to pS 5 2 ($6\ 3 \times 10^{-6}$ M)

Normal rats	[ChE] µmol/min /g brain	Tolerant rats	[ChE] µmol/min /g brain
1	0 47	1	0 46
2	0 51	2	0 52
3	0 55	3	0 55
4	0 59	4	0 60
5	0 60	5	0 72
[illegible]	0 63		
7	0 64		
8	0 71		
9	0 87		

2) In the presence of morphine

In this experiment we employed tolerant and non tolerant rats, which were given intraperitoneal injections of 600 700 or 780 mg/kg morphine The tolerant rats were put on experiment 20 hours after final daily administration of morphine and the experiment was carried out at room temperature (about 20°)

Excitation and convulsions were seen in most of the rats, often with subsequent respiratory arrest which was considered a sign of death We were interested in the primary toxic effects of morphine and the duration of the experiment was therefore limited to 90 minutes Some non tolerant rats survived the first 90 minutes, they were therefore removed from the experiment The experiment now involved 19 non tolerant and 16 tolerant rats (tables 2 and 3) Of the non tolerant 18 and 3 of the tolerant rats died within 60 minutes of administering morphine, for comparison the re ma ning 13 tolerant rat [illegible]

lar [illegible]

portion was meanwhile stored at −20 Activity measurements were then carried out The activity was measured first without any addition of morphine and then after sufficient morphine had been added to obtain a morphine concentration in the reaction vessel identical with that found in the brain on morphine determination (in tables 2 and 3 " before " and " after " respectively) As substrate we used acetylcholine at a concentration of $6\ 3 \times 10^{-6}$ M (pS = 5 2)

Morphine determinations showed 20–40 µg morphine per g tissue In

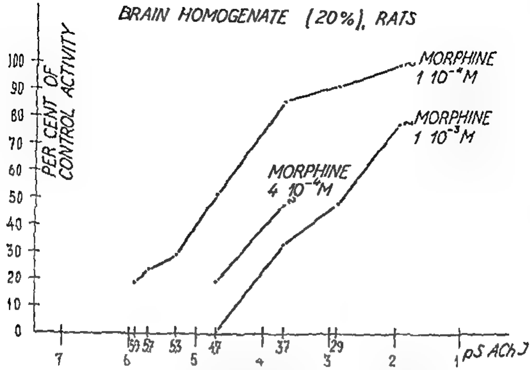

Fig 1 Homogenate (20%, w/v) of brains from 3 rats
Abscissa pS is the negative logarithm of the concentration of substrate, acetylcholine iodide (AChJ)
Ordinate The ordinate, expressed as percentage of the control activity, denotes the remaining cholinesterase activity after incubation for 10 minutes with morphine at three different molar concentrations

the activity to 20%, 40%, and 75%, respectively, whereas at pS = 4 7 it was reduced to 0%, 20% and 50% of the initial normal value

B Brain Cholinesterase Activity in Tolerant and Non-Tolerant Rats

1) In the absence of morphine

Without any preceding acute administration of morphine [illegible] non-tolerant and 5 morphine-tolerant rats (150–240 g) were killed, and [ChE] was determined in their brain homogenates without addition of morphine The substrate concentration was $6\ 3 \times 10^{-6}M$ It is seen from table 1 that [ChE] for the two groups is found in the same region (0 5–0 9 µmol/min /g)

The tolerant rats were killed 20 hours after the final daily dose of morphine For comparison another three tolerant rats were killed, also 20 hours after the final daily dose of morphine; their brains were homogenized, bulked and examined for morphine contents Analysis showed no measurable amounts of morphine

the brains of the tolerant rats we found an average morphine concentration of 30 3 µg/g, in the non tolerant rats 27 7 µg/g

[ChE] before addition of morphine ("before" in tables 2 and 3) was found to average 0 77 µmol/min /g in the tolerant and 0 68 µmol/min /g in the non tolerant rats After correction for the tissue dilution (i e morphine added to the reaction mixture up to a concentration identical with the concentration measured in the tissue) and after incubation for 10 minutes, activity measurings were made ("after" in tables 2 and 3) These measurements gave the mean values for [ChE] (i e the average remaining activities) in the tolerant rats of 0 42 µmol/min /g (~54 5%) and in the non tolerant rats of 0 40 µmol/min /g (~58 8%)

Before the addition of morphine [ChE] came up to the values given in table 1 and will be compared with these results Table 1 records the results of activity measurings with morphine absent in 5 tolerant and 9 non-tolerant rats Thus activity determinations were made in all on 21 tolerant and 28 non tolerant rats *without any addition of morphine*, and the mean [ChE] in the tolerant rats was 0 67 µmol/min /g and in the non tolerant rats 0 65 µmol/min /g

Discussion and Conclusions

It is necessary when measuring [ChE] to distinguish between reversible and irreversible cholinesterase inhibitors This distinction is observed in all enzymatic studies on these substances but its pharmacological and toxicological consequences have remained surprisingly unnoticed

Inhibition of cholinesterases by *irreversible inhibitors* (i e organic phosphorous compounds) results in the formation of stable compounds of enzyme and inhibitor, which can be split only under certain conditions. If on the other hand, it is a matter of inhibition by any of a number of *reversible inhibitors* (e g morphine) the conditions are entirely different, since both any dilution of the tissue and also addition of substrate will dissociate the initial compound of enzyme and inhibitor to a greater or lesser extent As all current methods for determining cholinesterase activity imply dilution of the tissue as well as addition of a substrate, most frequently at high concentrations, the results of such activity measurements will be misleading if the considerations mentioned are disregarded

In our experiments, on the basis of chemical determinations of morphine in samples taken from the precise brain tissue whose cholinesterase activity we wanted to examine we have added sufficient morphine to the reaction mixture to give a concentration identical with the concentration in the removed tissue We then had to decide which was the most appropriate substrate concentration for our purpose From fig 1 it will be seen

Table 2.

Non tolerant rats (19) given morphine 600–780 mg/kg intraperitoneally Brain cholinesterase activity, [ChE] is expressed as µmol/min /g "Before" means [ChE] *before* incubation

Number	Weight g	Morphine µg/g brain	[ChE] "before"	"after"	Remaining Activity in per cent	Dose mg/k	Time after Injection
K_{43}	160	19 2	0 64	0 45	70	780	20 min died
K_{45}	140	18 4	0 85	0 57	67	780	20 min died
K_{3}	260	26 4	0 50	0 29	58	780	22 min died
K_{41}	160	23 8	0 77	0 50	65	700	23 min died
K_{9}	160	27 6	0 52	0 30	58	700	25 min died
K_{52}	195	33 9	1 00	0 55	55	600	25 min died
K_{6}	260	27 2	0 48	0 28	58	700	26 min died
K_{10}	160	28 0	0 59	0 33	56	700	27 min died
K_{54}	145	28 6	1 02	0 55	54	780	28 min died
K_{40}	260	34 0	0 46	0 22	48	700	31 min died
K_{12}	160	36 2	0 66	0 29	44	700	34 min died
K_{39}	150	25 4	0 81	0 52	64	600	36 min died
K_{13}	180	20 1	0 53	0 36	68	600	36 min died
K_{42}	150	30 0	1 10	0 60	55	700	39 min died
K_{14}	180	27 2	0 63	0 35	56	600	42 min died
K_{47}	200	43 6	0 61	0 34(?)	56(?)	780	45 min died
K_{15}	180	23 6	0 51	0 33	65	600	53 min died
K_{16}	180	25 0	0 55	0 34	62	600	58 min died
K_{55}	200	27 6	0 71	0 42	59	780	90 min died

Table 3

Morphine tolerant rats (16) given morphine 600–780 mg/kg intraperitoneally For explanations, see table 2

Number	Weight g	Morphine µg/g brain	[ChE] "before"	"after"	Remaining Activity in per cent	Dose mg/kg	Time after Injection
T_{33}	140	36 9	1 01	0 46	46	780	25 min died
T_{4}	155	34 0	0 95	0 47	49	600	35 min died
T_{13}	155	25 1	0 75	0 50	67	700	35 min killed
T_{18}	145	31 1	0 80	0 43	54	700	35 min killed
T_{19}	140	19 4	1 04	0 69	66	700	35 min killed
T_{32}	160	31 0	0 53	0 28	53	780	40 min died
T_{21}	145	32 1	0 57	0 30	53	780	40 min killed
T_{22}	120	36 0	0 66	0 31	47	780	40 min killed
T_{23}	185	28 8	0 46	0 26	57	780	40 min killed
T_{24}	165	29 0	0 76	0 43	57	780	40 min killed
T_{5}	150	29 0	0 61	0 35	57	700	60 min killed
T_{6}	155	27 6	0 85	0 49	58	700	60 min killed
T_{7}	165	27 2	0 84	0 50	60	700	60 min killed
T_{8}	160	40 4	1 00	0 40	40	700	60 min killed
T_{1}	180	29 6	0 83	0 42	51	600	60 min killed
T_{2}	200	25 2	0 61	0 38	62	600	60 min killed

cholinesterase activity, as stated by KNOLL *et al* (1951) It further appears from the investigation mentioned that tissue dilution was not taken into account and that the substrate concentration used was extremely high

In rats' brain the amount of extractable acetylcholine will increase after injecting large doses of morphine (HERKEN *et al* 1957), and this increase can hardly be explained in terms of increased synthesis of acetylcholine (*cf* MORRIS 1961) It appears from our experiments that large doses of morphine will appreciably inhibit the cholinesterase activity of the brain, and it is well known that large doses of morphine, particularly lethal doses, will cause excitation and convulsions in both rats (JOËL & ETTINGER 1926) and rabbits (CORRADO & LONGO 1961), as well as in other species, but not in man (WOODS 1956, REYNOLDS & RANDALL 1957) It therefore seems highly probable that the excitation caused by large doses of morphine may be conditioned by cholinesterase inhibiting in the central nervous system, and JOHANNESSON & LAUSEN (1961) advance the argument that motor excitement after large doses of chlorpromazine may be similarly conditioned

Most of the non tolerant rats were dead 40 minutes after administering morphine, whereas most of the tolerant rats were still alive The tolerant rats were killed 35, 40, and 60 minutes respectively after the injection; we then found a mean concentration of morphine in their brains slightly higher than that of the non tolerant rats (tables 2 and 3) The question arose therefore, of a pronounced pharmacological tolerance, since the concentration was the same or higher, and the effect – expressed as lethality in our experiments – was slighter in the tolerant than in the non-tolerant animals It is probable that a centrally conditioned respiratory depression from acute morphine intoxication may be the direct cause of death in rats (*cf* SCHAUMANN 1958) The reduced lethality to the tolerant rats may therefore presumably be explained as a development of tolerance *towards* the depressing effect of morphine on respiration, as tolerance in a number of animal species (SEEVERS & WOODS 1953), including rats (JOËL & ETTINGER 1926 and KAYMAKCALAN & WOODS 1956), is mainly developed against the depressive effects of this substance On the other hand, experiments with dogs, rabbits and rats (SCHMIDT & LIVINGSTON 1933, GRINDT & HUESGEN 1937, GIBBS & BOBB 1938) seem to indicate that the occurrence of death from acute morphine poisoning is not caused by a progressive centrally conditioned respiratory paralysis, but seems to be associated with convulsions If excitation caused by lethal doses of morphine, observed in our experiments also, contributes much to the lethality, this would imply a development of tolerance *in* rats against the excitative effects, but the point of attack is presumably elsewhere than on the cholinesterases of the central nervous system (see below)

that the degree of inhibition after reversible cholinesterase inhibitors like morphine will rise with decreasing substrate concentrations The results of activity measurings will thus depend on the substrate concentration used It is therefore clear why LAVIKAINEN & MATTILA (1959) could not demonstrate any inhibition of rat brain cholinesterases after morphine injections, since they diluted the tissue 100 times without taking the morphine concentration into account and used a substrate (metacholine) of as high a concentration as 3×10^{-2}M

The dissociation, resulting from the addition of substrate, can be greatly reduced if acetylcholine is used at as low molar concentrations as 10^{-5}–10^{-6} We therefore decided to work with a substrate concentration of approximately 6×10^{-6}M On the basis of work by ACHESON (1948) and WASER (1960) JENSEN-HOLM (1962) later estimated the "physiological" concentration of acetylcholine to be approximately 10^{-6}M at the motor end-plate If we had used this substrate concentration, the remaining activities after incubation with morphine would have been both absolutely and relatively lower than those we in fact found (tables 2 and 3) It should, however, be emphasized that on the whole we have only an imperfect knowledge of the physiological environment (substrate, electrolytes, etc) that largely determine all cholinesterase activity in the central nervous system *in vivo* Further, homogenization will eliminate cell boundary lines, so that in the brain the cholinesterases, demonstrated by KOELLE and collaborators as "functional" and as "reserve" enzymes, (HOLMSTEDT 1959) will be artificially confounded Moreover, in our experiments the tissue dilution was often considerable (several hundred times) Assuming that we could only make a poor imitation of the physiological conditions under which the splitting of choline esters takes place naturally, we therefore used a 0 9% NaCl solution as diluent

After injecting large doses of morphine (600–800 mg/kg) into rats, we found 20–40 μg morphine per g brain in both tolerant and non-tolerant rats In the reaction vessel, however, the dilution was so considerable that activity determinations ("before" in tables 2 and 3) showed [ChE] as being of the same order as in the results given in table 1 It is obvious, therefore, that in our investigations, the activity measurements in presence of morphine, but without any correction for the tissue dilution employed, are fully comparable with the results obtained from activity measurements in the absence of morphine Having corrected for tissue dilution by adding morphine to the reaction mixture up to a morphine concentration identical with the concentration measured in the tissue, we found the activity reduced by 30–60% in both groups ("after" in tables 2 and 3) It is therefore a matter of considerable doubt whether morphine in small doses (e g 5–10 mg/kg) would be able to induce any appreciable inhibition of brain

determined, first without morphine and then by incubation for 10 minutes with such amounts of added morphine as would give morphine concentrations in the reaction mixture (organ homogenates with additions) identical with the concentrations in the tissues of the corresponding brains at death.

3 Applying acetylcholine iodide as substrate at molarity 6.3×10^{-6}, brain cholinesterase activity was found to be of the same order in morphine tolerant as in non tolerant rats In both groups also the activity was reduced by morphine to the same extent, *i e* by 30–60%

4 It is assumed that the excitation seen in both tolerant and non tolerant rats after large doses of morphine, may be explained *in terms of* cholinesterase inhibition in the central nervous system

5 The experimental results are discussed in relation to the phenomena of morphine tolerance in animals

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When identical substrate concentrations, with or without addition of morphine, are used, it seems a matter of great doubt whether there are any differences between brain cholinesterase activities in tolerant and non tolerant rats (tables 1, 2 and 3) The results of the experiments therefore argue in favour of the assumption that the ability of the brain to split acetylcholine is *the same* in tolerant as in non-tolerant rats and that in both groups, morphine will inhibit brain cholinesterases *to the same extent*

Investigators have tried to explain the development of morphine tolerance in animals by established differences between enzyme activities in tolerant and non-tolerant organisms In several studies, AXELROD (1956), AXELROD & COCHIN (1957), and COCHIN & AXELROD (1959) tried to parallel the defective ability of the liver microsomes to N-demethylate morphine in tolerant rats with the development of tolerance towards the analgetic effect of this substance We did not find this difference for cholinesterase, the ability of the brain to split acetylcholine under the given experimental conditions was the same in tolerant and in non-tolerant rats It should, however, be emphasized that at any given concentration of morphine the resultant from the compound of enzyme, inhibitor and substrate will be highly dependent on the amount of the last-mentioned (cf fig 1) Though there is every reason to believe that the two first mentioned factors may be present to the same extent *in vivo* in the brains of both tolerant and non-tolerant rats, we have no further knowledge of possible differences in the amounts of the last-mentioned factor The unchanged ability to split acetylcholine, which we found in our experiments, seems to indicate, but it does not prove, that the cholinesterase activity of the brain is the same in tolerant as in non-tolerant rats

In this connection it may be of interest to mention that TORDA & WOLFF (1946) and MORRIS (1961) found that morphine will to some extent reduce the synthesis of acetylcholine in the brain and SCHAUMANN (1956, 1957) demonstrated that morphine will inhibit the liberation of acetylcholine from nerve ends, but that the results of experiments confirming these assumptions are apparently not available for morphine-tolerant organisms

Summary.

1 Large doses of morphine (600, 700 or 780 mg/kg) were administered intraperitoneally to morphine tolerant or non-tolerant male albino rats The non-tolerant rats died between 20 and 60 minutes after being injected, for comparison the tolerant rats were killed at the same times

2 In the brains of rats of both groups the morphine concentration was found to be 20–40 µg/g Subsequently brain cholinesterase activity was

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nitrogen in the effluent solution from the ion-exchange column was measured by the ninhydrin colorimetric procedure of COCKING & YEMM (1954) The amino acids present in the effluent solution were separated by high voltage electrophoresis For this purpose use was made of the pherograph described by WIELAND & PFLEIDERER (1955) The buffer pH 1 2, consisted of glacial acetic acid formic acid and water (100 150 750 by vol) 20 µl solution was placed 6 cm from the anode end of a Whatman No 3 MM filter paper The running time was 3 hours at 50 volts/cm After electrophoresis the paper was dried for approximately 2 hours at a temperature of about +60 C It was then sprayed with a ninhydrin solution prepared by the [illegible]

[illegible] ously run to permit qualitative and semi-quantitative screening of the amino acids in the urine

The glycine areas were cut out and the copper complexes were eluted with ethanol The extinction was read in a Beckman spectrophotometer Model B at 504 mµ in a 1 cm cuvette against a corresponding paper blank

The concentration of protein in the urine was estimated by means of albustix ® (Ames analytical reagents)

The urinary lead was measured by the method of BESSMAN & LAYNE (1955) as slightly modified by HAEGER ARONSEN (1960)

The δ-aminolaevulic acid (ALA) in the urine was determined by the method of MAUZERALL & GRANICK (1956)

The creatinine in the urine was determined by the Jaffe reaction

Normal values found in a previous investigation (HAEGER ARONSEN 1960) for urinary ALA lead and creatinine in rabbits were used

Results

During the experimental period the rate of excretion of creatinine in the urine in the 7 lead poisoned rabbits was fairly steady (52–98 mg%, mean = 71) and never exceeded the upper normal limit The urinary α amino nitrogen ALA glycine and lead were therefore calculated per unit weight of creatinine in the samples

The mean excretion of α amino nitrogen in the urine in 10 healthy rabbits was 9 8 µmol/mg of creatinine (S D = 3 7) The corresponding figure for glycine was 2 3 µmol/mg (S D = 0 8)

During the first few days after administering the lead the excretion of the α amino nitrogen was increased (fig 1) It fell on the 4th–5th day, but rose again to reach a new maximum on the 8th 9th day The excretion was almost normal on the 10th–14th day after which it again rose continuously

Electrophoretic screening of the urinary amino acids before and at different intervals after administering lead showed that the glycine fraction varied most (fig 2) The concentration of histidine, like that of glycine was increased during the first 2 days and fell on the 3rd day,

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From the Department of Clinical Chemistry, University of Lund, General Hospital, Malmo, Sweden

Urinary Excretion of Amino Acids in Lead-Poisoned Rabbits

By

Wilfried von Studnitz and Birgitta Haeger-Aronsen

(Received November 14 1961)

In a related investigation of urinary porphyrins and their precursors in lead-poisoned rabbits the α-amino nitrogen was found to be moderately increased This increase, which was demonstrated already in the acute stage of the poisoning, coincided with the rise in excretion of δ-amino laevulic acid (ALA) noted in a previous study (HAEGER ARONSEN 1960)

An increased urinary excretion of amino acids in human chronic lead poisoning has been described by various authors (WILSON, THOMSON & DENT 1953, BICKEL & SOUCHON 1955, CHISOLM HARRISON, EBERLEIN & HARRISON 1955, CLARKSON & KENCH 1956) A search of the literature, however, failed to reveal any reports on the urinary excretion of amino acids during the *acute* phase of such a poisoning

Material and Methods

Urine samples from 10 full grown rabbits were used for determining the normal values for α amino nitrogen and glycine Seven of the rabbits (4 males 3 females), weighing between 2 5 and 3 5 kg afterwards received a single dose of 4% lead acetate subcutaneously in the back The dose corresponded to 125 mg of lead acetate per kg of body weight The animals were kept in metabolism cages and the urine was collected over periods of at least 15 hours without addition of preservatives

The concentrations of α amino nitrogen and creatinine in urine samples from 5 poisoned rabbits were determined before and on 18 subsequent occasions within 31 days of, injecting the lead acetate The concentrations of glycine, ALA lead and creatinine were each determined in urine samples from all the poisoned animals before, and on 9 occasions within 19 days of the injection Electrophoretic screening of amino acids was made in urine samples from these 7 rabbits before and on 7 occasions within 25 days of, the injection

The free amino acids were isolated from the urine by means of a sulphonated ion-exchange resin as recommended by CLARKSON & KENCH (1956) The α amino

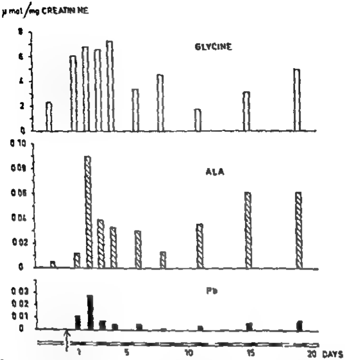


Fig 3 Mean urinary excretion of glycine δ aminolaevulic acid (ALA) and lead (Pb) in 7 rabbits Arrow indicates time each animal received lead acetate s c at a dose of 125 mg/kg b w

rising again continuously for the rest of the experimental period Screening on the 20th and 25th day after administering lead showed the concentrations of tyrosine and glutamic acid to be raised

During the first 15 days after injecting lead the concentration of serine was below normal in all of the animals On the 20th and 25th day the concentration of this amino acid was, if anything, increased

Fig 3 shows the mean excretions of glycine in the urines of 7 rabbits before and after administering lead The figure also shows the simultaneous mean excretion of ALA and lead in the urine

The excretion of glycine rose on the day after administering lead, on the 1st–4th day it reached a maximum corresponding roughly to twice the value of the upper limit of the normal range (mean + 2 S D) At the same time the urinary ALA and lead showed peaks corresponding roughly to 7 and 40 times the values of the upper normal limits

After these initial peaks the curves declined until about the 10th day and then rose continuously again

No proteinuria could be demonstrated at the time of the first glycine peak During the ascent of the glycine curve towards its second maximum, a moderate proteinuria was noticed

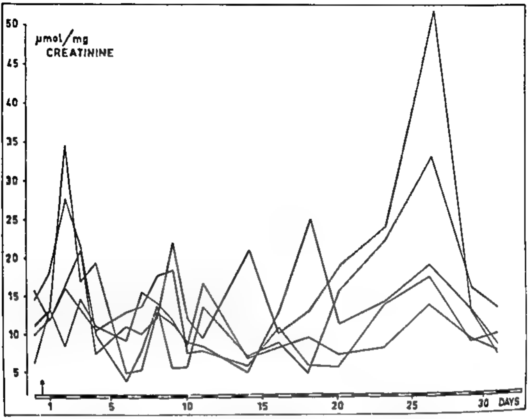
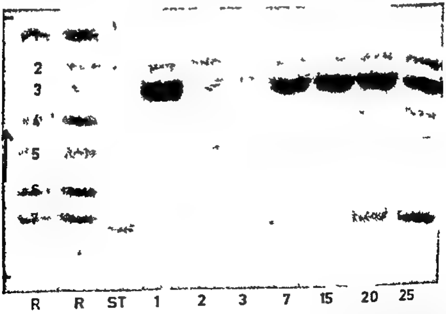


Fig 1 Urinary excretion of α amino nitrogen in 5 rabbits before and after administration of lead Arrow indicates time at which each animal received lead acetate s c in a dose of 125 mg/kg b w



F - -^ irinary amino acids in a rabbit before
(R – Amino acid reference standard
Serine 6 – Glutamic acid 7 – Tyrosine

It should also be mentioned that corticotropin has been shown to increase the excretion of glycine in human beings (ROBERTS RONZONI & FRANKEL 1951)

Earlier papers describing aminoaciduria in lead poisoning are based on chronic cases WILSON, THOMSON & DENT (1953), BICKEL & SOUCHON (1955) and CHISOLM, HARRISON, EBERLEIN & HARRISON (1955) described one patient each, all boys 2 to 4½ years old who after a long history of pica for painted things developed symptoms of lead poisoning The 3 boys had not only severe aminoaciduria due in the main to increased excretion of glycine β aminoisobutyric acid alanine, glutamic acid serine and threonine, but also glucosuria The boy described by CHISOLM *et al* had hyperphosphaturia as well This combination of signs localized the attack of lead in the kidneys to the proximal tubules where amino acids glucose and phosphate are normally re absorbed CLARKSON & KENCH (1956) found the concentration of total amino nitrogen in the urine to be increased in 6 of 27 workers exposed to lead Analysis showed the increase to be in the main due to alanine methylhistidine, tyrosine and threonine

The increased concentration of urinary amino acids observed in the rabbits 20–25 days after the administration of lead corresponded roughly to the type of aminoaciduria that might be expected in tubular injury

Summary.

On examining the excretion of urinary amino acids by lead poisoned rabbits changes were noted mainly in the amounts of δ aminolaevulic acid (ALA) glycine and serine during the acute stage of the poisoning The curves for the excretion of ALA and of glycine were biphasic, with an initial peak on the 1st–4th day after administering the metal The curves then fell to rise continuously again about 1 week later The initial ascent of the curves for excretion of the two amino acids was probably due to a prerenal disorder but the second rise was ascribable at least partly to tubular injury with decreased re absorption of amino acids

The excretion of serine fell initially but then rose again on the 20th–25th day after the administration of the lead It is suggested that the decreased excretion of serine combined with increased excretion of ALA and glycine in acute lead poisoning may be explained by blockage of ALA transaminase

Acknowledgement

We thank Miss Lis Britt Granberg and Mrs Mariann Nilsson for skilful technical assistance The work was supported by grants from the

Discussion.

The findings showed that the increased urinary excretion of glycine in the lead-poisoned rabbits was a major contributory cause of the increase noted in the excretion of α amino nitrogen

Of the urinary amino acids studied during the first week after administering the lead, the only one besides glycine and ALA found to be excreted in increased amounts in the urine was histidine

This abnormal excretion of amino acids in lead poisoning is probably ascribable to a disorder in prerenal metabolism or renal injury resulting in impaired re absorption or both

Judging from a preliminary analysis, the initial increase in the excretion of glycine is accompanied by an elevation in plasma glycine Corresponding findings have been made for ALA (HAEGER-ARONSEN 1960) This suggests a prerenal disturbance in metabolism of the two substances No simultaneous proteinuria was noted, so that tubular injury appears less likely in this stage of the poisoning

During the later rise impaired re absorption is probably a contributory cause of the increased excretion of glycine and ALA, for the excretion of other amino acids such as histidine, glutamic acid and serine, was then also increased

The mechanism by which the lead interferes with the metabolism of glycine is not properly understood Lead has long been known to be a powerful enzyme poison The simultaneously increased excretions of glycine and ALA, combined with a decreased excretion of serine, suggest a disturbance in the synthesis of porphyrins For glycine and succinyl CoA are condensed to ALA (SHEMIN & RUSSELL 1953), which is involved partly in the synthesis of porphobilinogen and subsequent formation of porphyrins and partly in a cyclic process during which the α carbon atom of glycine can be used in the synthesis of various substances requiring one carbon fragments (SHEMIN 1955) This process includes transamination (KOWALSKI, DANCEWICZ & SZOT 1957) after which there is a further conversion into succinate plus a one carbon fragment which is used for, among other things the synthesis of serine (GATT & SHEMIN 1955)

Partial blockage of ALA transaminase which catalyzes the above mentioned transamination (KOWALSKI DANCEWICZ & SZOT 1958) should presumably decrease the excretion of serine and increase the excretion of ALA and glycine

Since the possibility of enzymatic interference in other biochemical reactions in which glycine is an essential component cannot be excluded, the increased excretion of glycine and ALA and the decreased excretion of serine in the acute stage of lead poisoning need not be interdependent

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Effect of Temperature on the Action of Reserpine

By

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(Received November 13 1961)

In several recent papers the influence of ambient temperature on the action of reserpine has been reported LESSIN & PARKES (1957 a, b) found that reserpine produced in mice no apparent sedation at ambient temperatures of 32°C or more, when the usual fall of body temperature caused by reserpine was prevented GARATTINI & VALZELLI (1958) reported that previous exposure of the rat to heat (37°C) or cold (0°C) abolished the sedative action of reserpine as well as its depletion of brain 5 hydroxytryptamine (5 HT) SULSER & BRODIE (1960) and BRODIE *et al* (1960) made similar observations on the effect of cold (+4°C), further, they stated that the liberating effect of reserpine on brain noradrenaline persisted after exposure to cold According to BRODIE and co workers these observations support the view that the sedative action of reserpine is to be ascribed to the release of brain 5 HT rather than of noradrenaline Other investigators have stressed the role of the catecholamines in this connection (CARLSSON *et al* 1957, CARLSSON 1959, PLETSCHER *et al* 1959, KARKI & PAASONEN 1959)

In the present investigation attempts were made to reproduce the results mentioned above

Experimental Methods

Rats weighing about 200 to 250 g were used, generally male albino animals They were exposed to cold (+4°C or −10°) or *heat (+37°)* for various periods, up to 16 hours Reserpine at a dose of 1 0 or 2 5 mg per kilogram was then given intraperitoneally After a further 4 hours in the same environment the rats were killed, and their brains were analysed for 5 HT contents The time of exposure to heat had to be shortened somewhat, because the animals became exhausted and could not tolerate the heat effect for more than 6 to 7 hours in all

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Reserpine lowered the 5 HT content of the brain considerably

No obvious difference in 5 HT content was noticed as a result of the thermal changes, whether among the reserpine treated animals or among those not treated In the two reserpine groups the values for the heat- or cold pretreated rats seemed somewhat lower than those for the animals kept at room temperature, but the reverse was true of the two non-reserpinized groups The differences were not statistically significant

No certain differences in the behaviour of the animals could be detected between the reserpine treated groups All the animals treated with reserpine were sedated, with ptosis and characteristic hunched posture, irrespective of thermal environment

Discussion

The experiments were planned in such a manner as to permit direct comparisons with investigations performed earlier (GARATTINI & VALZELLI 1958, BRODIE *et al* 1960) The same reserpine doses and temperature conditions and the same time intervals were used except that the time for the heat pretreatment had to be reduced somewhat owing to the animals' exhaustion

The results obtained have not confirmed the conclusions of the previous reports that heat or cold pretreatment prevents the sedative or 5 HT-liberating effects of reserpine Nor could these preventive actions be reproduced by varying certain experimental conditions such as the sex and strain of the rats or the duration and intensity of cold exposure

A noteworthy circumstance was the relatively large fluctuations of brain 5 HT content from one experiment to another, even in the wholly untreated control animals These variations are statistically significant ($P < 0.005$ from an analysis of variance) Methodological errors do not seem to account for these variations since "internal standards" were used in the analysis for each single determination, with only small variation [illegible]

[illegible] of the animals throughout the twenty four hours The experiments described above were, however, all carried out at approximately the same time of the day after the reserpine injection Seasonal variations are also conceivable in view of the varying duration and intensity of daylight Among other factors having a possible influence are age sex and body weight

The 5 HT concentrations of the control animals, 0 59 to 0 93 µg per g brain tissue with mean values of 0 71 µg per g were somewhat higher

In each experiment one group of animals was treated in the manner described above, while another group received no thermal treatment. As additional controls were used both normal rats without treatment and rats exposed to heat or cold respectively, but not to the action of reserpine. Within each of these four groups as a rule two pooled rat brains were analysed. The animals' behaviour was observed during the experiments.

Analysis of the brains for 5-HT was carried out by the method of BERTLER (1961) slightly modified. The brains were rapidly removed and homogenized in ice cold 0.4 M perchloric acid, and the homogenates were then centrifuged. The subsequent determination of 5-HT in the supernatant extracts involves chromatographic separation of the amine from interfering substances on the cation exchange resin Amberlite XE 64. Elution was with a small volume of N-HCl. The final determination of the amine was carried out as described by UDENFRIEND *et al* (1955). The concentration of HCl in the eluate was made 3 N by addition of 12 N HCl and the fluorescence was read at 550 mμ with the activating wave length at 300 mμ (uncorrected instrumental values).

In all individual determinations the recoveries were checked by adding a known amount of 5-HT to one half of each homogenate. The recoveries ranged from 70 to 85%, the values given in table I have been corrected accordingly.

It is important that the time between homogenizing and centrifuging should be as short as possible. It should not exceed 30 minutes, after which recovery becomes lower.

5-HT determinations carried out by this method and by that of BOGDANSKY *et al* (1956) on the same brain homogenate agreed to within 10%.

Results.

The different experimental conditions and the results are given in table I.

Table I

Effect of Reserpine on Brain 5-HT in Rats at Various Ambient Temperatures

Strain	Sex	Temp °C	Time (before + after reserpine) hours	Reserpine dose mg/kg	Brain 5-HT μg/g: Reserpine, Room temp	Reserpine, Cold or heat	No reserpine, Room temp	No reserpine, Cold or heat
Albino	M	+ 4	16 + 4	1	0.37	0.28	0.69	–
Albino	F	+ 4	16 + 4	1	0.42	0.37	0.79	0.88
Black and white	M	10	9 + 4	1	0.46	0.50	0.79	0.76
Black and white	M	10	9 + 4	2.5	0.20*	0.22	0.59	0.66*
Albino	M	+37	3 + 3	2.5	0.48*	0.33*	0.93*	0.99*
Albino	M	+37	3 + 3.5	2.5	0.16	0.14	0.59	0.65

The number of rat brains in each group was 2 except in those groups marked*, in which only 1 brain was analysed.

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From the Department of Pharmacology University of Helsinki

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By

M Mattila

(Received January 8 1962)

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Material and Methods

Male albino rats weighing abo[illegible] 150 g were allowed to fast over [illegible]

[illegible] pilocarpine chloride barium chloride and 5-hydroxytryptamine creatinine sulphate Morphine chloride and nalorphine bromide were added in the bath fluid 30 seconds *before stimulation* The *degree of inhibition* is here expressed as ratios of doses giving an equal response with and without an antagonist

$$\frac{\text{agonist} + \text{antagonist}}{\text{agonist}}$$

than the "normal" value given by BRODIE *et al.* (1960), i. e. 0 45 µg per g. This difference may be at least in part due to the fact that the former value but possibly not the latter has been corrected for recovery. GARATTINI & VALZELLI (1958) reported normal values of about 0 90 µg per g, by a less specific fluorimetric method.

It thus seems essential always to run control animals in parallel with the test animals, otherwise the variations in normal value may be the cause of serious errors.

Summary.

The observation by other workers, that exposure of rats to cold or heat prevented the action of reserpine on behaviour and brain 5-HT, could not be confirmed.

Because of variations in normal brain 5-HT from one experiment to another, the importance of running control and test animals in parallel is emphasized.

Acknowledgements.

This work has been supported by a grant from the Medical Faculty, University of Göteborg, Sweden.

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Material and Methods

Male albino rats weighing about 150 g were allowed to fast over night before being killed Pieces of the small intestine were taken 15–20 cm proximally from the caecum. The preparation was stimulated at 25°C in a fluid bath (volume 5 ml) containing rat colon Ringer s solution (NaCl 9 0 KCl 0 4 $CaCl_2$ 0 03, $NaHCO_3$ 0 15 glucose 1 0 and aqua dist 1000 0) Stimulation was at 6 min intervals The *drugs* used were nicotine bitartrate pilocarpine chloride barium chloride and 5-*hydroxytryptamine* creatinine sulphate Morphine chloride and nalorphine bromide were added to the bath fluid 30 seconds before stimulation The *degree of inhibition* is here expressed as ratios of doses giving an equal response with and without an antagonist

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agonist

Table I.

The analgesic effect of morphine on tolerant and non tolerant rats
Time in seconds from first stimulus to withdrawal of tail

	Reaction time											
	Tolerant rats							Controls				
Before morphine	12	10	20	8	18	5	16	23	9	13	11	8
After morphine (50 mg/kg i p)	22	15	30	28	45	25	29	>180	>180	>180	>180	>180

Tolerance and addiction were produced by injecting doses of morphine twice daily for 3 weeks Initially the injections were subcutaneous, later intraperitoneal because of the large volumes of morphine solution needed and the changing absorption from the subcutaneous tissue (MILTHERS 1959) Morphine was given to 7 rats, while 5 controls received a similar amount of saline

Criteria for tolerance 1) At the end of the 3-week period the rats withstood lethal doses of morphine chloride (600 mg/kg twice a day)

2) The analgesic effect of morphine decreased as tolerance developed The tails of the rats, treated with ether and electrode jelly, were stimulated by electric impulses (70 v, 20 Hz), and the time from the first impulse to the withdrawal of the tail was measured The differences between the tolerant and normal rats can be seen from table I Before the morphine treatment all rats reacted in the same way as the control rats at the end of the 3-week period

3) *Abstinence symptoms* were provoked by nalorphine (15 mg/kg i p) at the end of the third week (HANNA 1960 The rats became restless and quarrelsome 1–2 min after injection and showed a positive masseter symptom, hyperpnoea and diarrhoea Neither the rats given morphine nor the control rats showed any symptoms Morphine (500 mg/kg i p) eliminated the symptoms within 10 min

Four guinea-pigs also received 30–100 mg/kg morphine chloride subcutaneously once daily for 3 weeks At the end of this period, the analgesia induced by morphine was decreased Electric (50 v, 2 Hz) impulses were transmitted to the interscapular region via needle electrodes (WEGER & AMSLER 1936) Guinea-pigs normally react to each impulse with a squeal "Complete analgesia" is taken to mean 100 successive impulses without a squeal After morphine (20 mg/kg), the tolerant guinea-pigs squealed after 1–6 impulses, whereas the control animals tolerated 100 impulses without squealing The guinea-pig ileum was stimulated at 37° in Tyrode's solution in the same way as the rat intestine

Results.

A. The Normal Intestine

The rat intestine is comparatively resistant to morphine. There was no effect from 10 µg but 100 µg inhibited the nicotine contractions weakly and 500 µg strongly The last-mentioned dose corresponds to a concentration of morphine of 10^{-4} Morphine increased the tone of the intestine

Table 2

The inhibitory effect of morphine and nalorphine on the contractions of the non tolerant rat intestine The antagonism is expressed as a ratio of doses $\frac{\text{agonist} + \text{antagonist.}}{\text{agonist}}$

Spasmogen	Morphine 10^{-4}	Nalorphine 2×10^{-5}	Morphine 10^{-4} Nalorphine 10^{-5}
5 HT	1 2, 1 1 5, 1	2, 2 3, 2	1, 2, 1 5
Nicotine	6, 3 8, 4	10 8, 3 5, 6	10, 7
Barium	2, 1 8 1 5	2 5 2 5 3	2 2 5
Pilocarpine	1 1 2 1 2 1	1 8 2 2 5	2 3, 1 3 3

The inhibitory action on the contractions caused by various drugs is shown in table 2 Morphine inhibited nicotine contractions strongly, but those caused by 5-HT and pilocarpine only slightly (fig 1, 2 and 3) The ileum and the jejunum reacted similarly to different stimulants

Nalorphine did not increase the intestinal tone, on the contrary, its effect could be relaxing fig 2 and 3) Doses of 100–300 μg (final concentrations 2–6 $\times$ 10^{-5}) prevented contractions caused by various drugs Table 2 shows that nalorphine had a stronger inhibitory effect than morphine on all contractions, including those caused by pilocarpine This implies either a more potent effect or a more "distal" site of action

The effect of morphine and nalorphine combined showed an inhibitory effect on various contractions similar to that of nalorphine alone (table 2) No mutual antagonism could be demonstrated in the rat intestine with the technique used

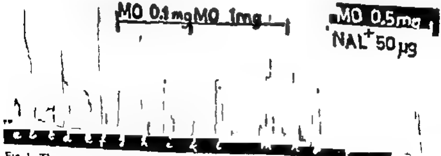

Fig 1 The nicotine and 5 HT contractions in the small intestine of the non tolerant rat. Doses of *5 HT* a, c 0 2 μg e, g i, l, n, r 0 4 μg p 0.5 μg Doses *of nicotine* b 20 μg, d 15 μg, f, h, k, o 25 μg m 95 μg q 60 μg
Morphine is a poor inhibitor to the 5 HT contractions

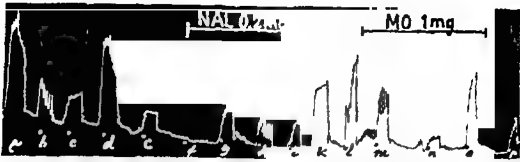

Fig 2 The effect of morphine and nalorphine on contractions of the small intestine of the non tolerant rat Doses of stimulants *pilocarpine* a, g 15 µg, d 12 µg, k, m 10 µg *Barium* b 500 µg h 1 o 750 µg *Nicotine* c, f 150 µg, e, i, n p 100 µg
Nalorphine is a more potent inhibitor than morphine

B The Tolerant Intestine

Possible withdrawal symptoms (increased sensitivity or unusual response to nalorphine) in the isolated intestine were studied

Morphine and nalorphine prevented contractions caused by various drugs, just as they did in the normal intestine The inhibitory dose of morphine needed was usually twice as large as for the normal intestine The intestines of 4 rats showed an abnormal sensitivity to nalorphine a dose of 100–200 µg provoked a strong relaxation (fig 3), which was counteracted by a large dose of morphine In the other three intestines this did not appear Thus abstinence symptoms could not be confirmed in the isolated rat intestine

The intestine of the tolerant guinea-pig was less sensitive to morphine than that of the normal guinea-pig To prevent nicotine contractions, 10–50 times larger doses of morphine (100–500 µg pro 10 µg) were required Inhibition of the 5-HT contractions was also weaker than in the intact intestine In neither intestine was there any inhibition of histamine contractions The ileum of the tolerant guinea-pig showed no abnormal sensitivity to nalorphine No abnormal tonic effect on the intestine of the

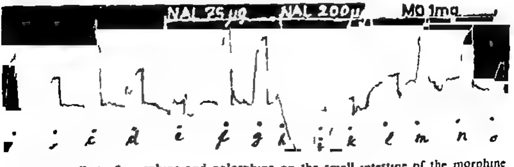

Fig 3 The effect of morphine and nalorphine on the small intestine of the morphine ... h 1 o 100 µg

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tolerant guinea pig (LEVY & CAHEN 1933) could be observed in these experiments The intact intestine was more sensitive to nicotine stimulus after morphine but not after nalorphine

Discussion.

The small intestine of the rat showed some characteristic reactions

1) Nalorphine did not prevent the inhibitory action of morphine on nicotine-induced contractions In the guinea-pig ileum, however, nalorphine [illegible] able to abolish the inhibitory effect of morphine (KOSTERLITZ & ROBINSON 1958) Because nalophine also prevents pilocarpine contractions more effectively than does morphine it may be assumed that nalorphine has a different site of action from that of morphine Nalorphine acts more peripherally, perhaps directly on the smooth muscle cell Some antagonistic action between morphine and nalorphine was demonstrated in the tolerant intestine, where morphine increased the tone of the intestine depressed by nalorphine It is difficult to say whether this antagonism is specific or nonspecific

2) M receptors (GADDUM & PICARELLI 1957) could not be demonstrated so clearly in the rat intestine as in the guinea pig's Nor does morphine prevent the 5-HT contractions in the mouse intestine (unpublished observations) This may be because the rat intestine preparation is not sufficiently sensitive to 5-HT Another possible explanation is that the site of effect of 5-HT is more distal than that of morphine, but is more easily reached by nalorphine

Morphine caused an abnormal relaxation in the intestine of some tolerant rats (fig 3), where the nicotine contractions were prevented This phenomenon was not observed in the intestine of all tolerant rats, though [illegible] tolerance to morphine was established The tolerance was marked [illegible]

a [illegible]

s [illegible] primarily in the central nervous system and not in the peripheral organ It is not clear, however, whether or not the tolerance in the intestine was equal to that in vivo, a finding that has been questioned earlier (MILLER & PLANT 1926) It is possible, too, that the acquired tolerance mechanisms were disturbed by the administration of nalorphine and that the intestine did not react as an "addicted" intestine should do

Summary.

The isolated intestines of the normal and the morphine tolerant rat and guinea pig were stimulated with nicotine, pilocarpine, barium, 5-HT and

histamine. The action of morphine and nalorphine on these contractions was studied.

1) Morphine is an effective inhibitor of nicotine contractions in the rat intestine, a poor inhibitor of those caused by 5-HT and pilocarpine. Nalorphine prevents all these contractions more effectively than does morphine.

2) No morphine-nalorphine antagonism was demonstrated in the rat intestine.

3) The intestine of the "addicted" rat was extremely sensitive to nalorphine in individual animals. This phenomenon was not observed in the intestine of the tolerant guinea-pig, which showed a marked tolerance to morphine.

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Scha [illegible] -vende [illegible] *exp*

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From the Research Laboratories of the State Alcohol Monopoly (Alko), Helsinki Finland (Dr Heikki Suomalainen)

Changes in Respiration of Rat Brain Cortex Slices Induced by some Aliphatic Alcohols

By

Ralph Lindbohm and Henrik Wallgren

(Received January 22 1962)

During studies in these laboratories of the action of ethyl alcohol on brain cortex tissue stimulated electrically *in vitro* by a method developed by McIlwain (1951), it seemed desirable to obtain information about the effects of other closely related aliphatic alcohols Beer & Quastel (1958) have reported results for brain tissue stimulated with potassium ions, – a preparation, less sensitive, however, to alcohol than electrically stimulated tissue (Wallgren & Kulonen 1960)

Ferguson (1939) pointed out that there exists a large and heterogeneous group of "physical" anaesthetics, including the aliphatic alcohols, which display narcotic activities correlating roughly with their thermodynamic potentials or "activities" This relationship was later supported by the experiments of Brink & Posternak (1948)

The thermodynamic activity" of a compound is a measure of its partial molal free energy According to Brink & Posternak (1948), an absolute scale for the notion has not been established, but changes in energy during the reversible transfer of a substance from one state to another at the same temperature have been calculated and give a measure of the molal free energy This reflects the work performed in the transfer between any two phases and thus also the distribution, for instance, between a pure liquid and air or a water phase in contact with a lipoid phase Ferguson (1939) and Brink & Posternak (1948) have based their investigations on the reasonable assumption that many narcotic agents reach an equilibrium in every part of the system in which the narcosis occurs The thermodynamic activity of a compound at equilibrium in a system is then the same in all its phases Thus if the concentration of the narcotic in the water phase of the system, and its activity coefficient, f, are known, then its activity in the phase where the narcosis is induced can be

calculated (For equations, see BRINK & POSTERNAK 1948) Especially for the members of a homologous series, such as the normal aliphatic alcohols, equal narcotic potency has been shown to occur at the same thermodynamic activity (BRINK & POSTERNAK 1948)

In a way this concept is a reformulation in more general terms of the MEYER-OVERTON lipoid theory of narcosis This theory has recently received support from experiments performed by SKOU (1958), who showed that the abilities of several narcotic compounds to penetrate a monomolecular layer of nerve-tissue lipoid are closely correlated with their powers to block impulse transmission in peripheral nerves In view of this, we decided to make the molar concentrations of the alcohols employed inversely proportional to their thermodynamic activities, using as reference point ethyl alcohol at a concentration causing intoxication but not death in the intact animal It was expected that the effects consequently obtained would be of about the same magnitude

Material and methods

All experiments were performed in conventional Warburg constant-volume respiratory manometers Oxygen uptake was used as a measure of the activity of the brain tissue

A detailed description of the apparatus for electrical stimulation, which was similar in principle to that developed by MCILWAIN (1951) has been given elsewhere (WALLGREN & KULONEN 1960) A phosphate glucose medium (MCILWAIN 1951) containing 6 mM glucose was used in all experiments, the temperature being 37 5°C, and the gas phase was pure oxygen Adult rats from the laboratory stock were used In all experiments, 2 standard and 2 electrode flasks were used From each brain hemisphere three slices were cut one slice (20–30 mg) was used for determining the respiration of unstimulated tissue and two slices (40–60 mg) that of stimulated tissue The slices from one hemisphere were employed as controls for those from the other, which were exposed to alcohol A volume of solution (0 2 ml) containing the required amount of alcohol was pipetted into the side arm of each flask after these had been flushed with oxygen and tipped into the main compartment after about 10 min equilibration The alcohols used in these experiments are listed in table 1 The ethanol was Alko Aas (for spectrophotometric work) the others Merck's *pro analysi* grade The concentration of ethanol used was 0 130 M (0 6%) which from experience was considered high enough to give a marked effect, yet would correspond to "physiological" conditions representing ca 2/3 of the blood concentration lethal to rats The molar concentrations of the other alcohols were inversely proportional to their thermodynamic activities relative to that of ethanol

In the statistical evaluation of the results the t test for correlated measures was applied to the values for the difference between control and experimental tissue in each alcohol group, since in each experiment the slices were from the same brain Student's t test was employed for comparing the effects of the different alcohols

Table 1

Thermodynamic activity coefficients (f from BUTLER 1935 according to BRINK & POSTERNAK 1948) of the alcohols used and their molar concentrations (M) in the incubation medium for the brain slices

Alcohol	f	M
Ethyl	3 69	0 130
n Propyl	14 4	0 033
*Iso*propyl	7 7	0 062
n-Butyl	52 9	0 009
t Butyl	11 8	0 041

Results

Ethyl alcohol at the concentration employed reduced the respiration of stimulated tissue by 11 5% (table 2) The effects of the other alcohols were close to this value The slightly higher figure obtained for butyl alcohol (15 9%) did not differ significantly from those obtained with the other alcohols

No such uniformity of action was found in unstimulated tissue (table 2) Ethyl alcohol and *n* propanol caused significant increases in respiration

Table 2

Respiration of the brain cortex tissue in various experimental conditions μmoles O_2/g fresh tissue/hr mean values (standard deviations) The P values indicate the significance of the differences in effect of the alcohols

Alcohol	Unstimulated tissue				Stimulated tissue			
	No of expts	Control	Alcohol	*Per cent change* (P <)	No of expts	Control	Alcohol	Per cent change (P <)
Ethyl	12	129 9 (13 0)	138 8 (9 4)	+ 6 9 (0 05)	13	199 1 (12 1)	176 3 (9 8)	−11 5 (0 001)
n Propyl	11	124 3 (23 1)	138 4 (20 0)	+ 10 2 (0 02)	8	196 8 (17 6)	174 5 (13 8)	−11 4 (0 01)
*Iso*propyl	8	128 2 (11 4)	123 0 (14 3)	−4 2 (not sign)	8	195 4 (11 6)	170 7 (11 6)	−12.6 (0 001)
n Butyl	12	144 7 (9 3)	133 9 (14 1)	−7 5 (0 01)	10	208 3 (11 6)	182 3 (18 8)	−12 5 (0 005)
t Butyl	10	131 5 (7 7)	120 3 (13 7)	−8 5 (not sign)	11	202 1 (22 8)	170 0 (14 2)	−15 9 (0 005)

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Material and methods

All experiments were performed in conventional Warburg constant volume respiratory manometers Oxygen uptake was used as a measure of the activity of the brain tissue

A detailed description of the apparatus for electrical stimulation which was similar in principle to that developed by MCILWAIN (1951) has been given elsewhere (WALLGREN & KULONEN 1960) A phosphate glucose medium (MCILWAIN 1951) containing 6 mM glucose was used in all experiments the temperature being 37 5°C and the gas phase was pure oxygen Adult rats from the laboratory stock were used In all experiments 2 standard and 2 electrode flasks were used From each brain hemisphere three slices were cut one slice (20–30 mg) was used for determining the respiration of unstimulated tissue and two slices (40–60 mg) that of stimulated tissue The slices from one hemisphere were employed as controls for those from the other which were exposed to alcohol A volume of solution (0 2 ml) containing the required amount of alcohol was pipetted into the side arm of each flask after these had been flushed with oxygen and tipped into the main compartment after about 10 min equilibration The alcohols used in these experiments are listed in table 1 The ethanol was Alko Aas (for spectrophotometric work) the others Merck s *pro analysi* grade The concentration of ethanol used was 0 130 M (0 6%) which from experience was considered high enough to give a marked effect yet would correspond to physiological conditions representing ca 2/3 of the blood concentration lethal to rats The molar concentrations of the other alcohols were inversely proportional to their thermodynamic activities relative to that of ethanol

In the statistical evaluation of the results the t test for correlated measures was applied to the values for the difference between control and experimental tissue in each alcohol group since in each experiment the slices were from the same brain Student s t test was employed for comparing the effects of the different alcohols

Table 1

Thermodynamic activity coefficients (f, from BUTLER 1935 according to BRINK & POSTERNAK 1948) of the alcohols used and their molar concentrations (M) in the incubation medium for the brain slices

Alcohol	f	M
Ethyl	3.69	0.130
n Propyl	14.4	0.033
*Iso*propyl	7.7	0.062
n Butyl	52.9	0.009
t Butyl	11.8	0.041

Results.

Ethyl alcohol at the concentration employed reduced the respiration of stimulated tissue by 11.5% (table 2). The effects of the other alcohols were close to this value. The slightly higher figure obtained for butyl alcohol (15.9%) did not differ significantly from those obtained with the other alcohols.

No such uniformity of action was found in unstimulated tissue (table 2). Ethyl alcohol and *n* propanol caused significant increases in respiration

Table 2

Respiration of the brain cortex tissue in various experimental conditions. µmoles O_2/g fresh tissue/hr: mean values (standard deviations). The P values indicate the significance of the differences in effect of the alcohols

Alcohol	Unstimulated tissue				Stimulated tissue			
	No of expts	Control	Alcohol	Per cent change (P <)	No of expts	Control	Alcohol	Per cent change (P <)
Ethyl	12	129.9 (13.0)	138.8 (9.4)	+ 6.9 (0.05)	13	199.1 (12.1)	176.3 (9.8)	−11.5 (0.001)
n Propyl	11	124.3 (23.1)	138.4 (20.0)	+10.2 (0.02)	8	196.8 (17.6)	174.5 (13.8)	−11.4 (0.01)
*Iso*propyl	8	128.2 (11.4)	123.0 (14.3)	−4.2 (not sign.)	8	195.4 (21.6)	170.7 (11.6)	−12.6 (0.001)
n Butyl	12	144.7 (9.3)	133.9 (14.1)	−7.5 (0.01)	10	208.3 (11.6)	182.3 (18.8)	−12.5 (0.005)
t Butyl	10	131.5 (7.7)	120.3 (13.7)	−8.5 (not sign.)	11	202.1 (22.8)	170.0 (14.2)	−15.9 (0.005)

Iso propyl alcohol did not exert any clear-cut effect, whereas *n* butyl and *t*-butyl alcohol both depressed the respiration

With both stimulated and unstimulated tissue the alcohols were uniform in action throughout the one-hour experimental period

Discussion.

In table 3 is shown the striking parallelism between the action of the alcohols on all the *in vitro* preparations examined and their thermodynamic activities The intoxicating effects observed in rats also fit into this pattern reasonably well, considering the complexities involved in experiments performed with intact animals As pointed out by FERGUSON (1939) and recently by PAULING (1961), the close correlation with the thermodynamic activities does not give any clue to the actual mechanism of these compounds' action The data would fit any hypothesis attributing to intermolecular forces the decisive role in the induction of narcosis From this point of view it is worth stressing that, like the results quoted from SKOU (1958) in table 3, our results in all probability reflect interference with the response to stimulation of nerve cells Since the membrane structure is primarily involved in impulse conduction of excitable tissues, our results are also compatible with the idea that the site of the anaesthetic action of alcohols is located in these membranes Various authors have recently been led to the same tentative conclusion on the basis of different kinds of biochemical, electrophysiological and pharmacological evidence (QUASTEL & QUASTEL 1961, JARNEFELT 1961, KNUTSSON 1961, WALLGREN 1961) It also seems reasonable to suppose that the effect is brought about by the alcohol molecules themselves and not by their metabolites

Table 3

Relative effects of some aliphatic alcohols in four different test systems compared with their relative thermodynamic activities [illegible]

Colum[illegible] [illegible]ble 1

Alcohol	1 Thermo dynamic activity coefficient	2 Inhibitory potency stimulated tissue	3 Blocking potency peripheral nerve	4 Penetrating potency nerve lipoid	5 Molar intoxicating effect rats
Ethyl	1	1			1
n Propyl	3 9	3 9	3 9	3 9	2 5
*Iso*propyl	2 1	2 3	2 3	2 3	2 7
n Butyl	14 4	15 7	12 9	11 7	6 3
t Butyl	3 2	4 4			4 8

Any interpretation of the meaning of the difference between the results obtained with the stimulated and the unstimulated preparations must, with the information available, be highly hypothetical Perhaps the alcohols at this level of concentration act mainly by interfering with systems intimately linked to the excitation cycle of conducting membranes However, it cannot be concluded that the phenomena observed in unstimulated tissue are irrelevant for an understanding of the effects in stimulated tissue POSTERNAK & MANGOLD (1949) showed that methanol, ethanol and the propanols depolarized peripheral nerves whereas the butanols had no clear-cut effects on the membrane potential, and higher homologues hyperpolarized the membrane The different effects of these compounds on the respiration of unstimulated tissue might reflect their differing actions on the resting membrane

Summary.

The effects of the ethyl, *n* propyl, *iso*propyl, *n*-butyl, and *t* butyl alcohol at "physiological" concentrations (130, 33, 62, 9 and 41 mM, respectively) on the respiration of electrically stimulated and unstimulated slices of rat-brain cortical tissue were studied In the stimulated tissue, respiration was depressed by about 12% the effects of the alcohols being in close agreement with their thermodynamic activities In unstimulated tissue the effects were variable These results are interpreted as indicating that the alcohols at the concentration levels employed act primarily by interfering with mechanisms closely related to the excitation cycle in conducting membranes

Acknowledgements

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Table 3

Relative effects of some aliphatic alcohols in four different test systems compared with their relative thermodynamic activities

Column 1
2
3
5

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Ethyl	1	1			1
n Propyl	3 9	3 9	3 9	3 9	2 5
*Iso*propyl	2 1	2 3	2 3	2 3	2 7
n Butyl	14 4	15 7	12 9	11 7	6 3
t Butyl	3 2	4 4			4 8

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From the Research Laboratories of the State Alcohol Monopoly (Alko), Helsinki Finland (Dr Heikki Suomalainen) and the Psychological Institute University of Helsinki, Helsinki Finland (Prof Kai von Fieandt)

The Effect of Ethyl Alcohol on a Conditioned Avoidance Response in Rats

By

Henrik Wallgren and Sinikka Savolainen

(Received January 8 196?)

Although the conditioned avoidance response (CAR) has been widely used in studies on drug effects (cf e g HERZ 1960), little has been published about the effect of ethanol on this type of reaction Using WARNER's (1932) shuttle box, as modified by GELLHORN, KESSLER & MINATOYA (1942) BATTIG & GRANDJEAN (1955) found that the reaction time of rats was significantly increased after administering 2 ml (1 6 g) alcohol/kg body weight HOLTEN & SONNE (1955), using the same type of apparatus, did not study the CAR *per se*, but the manifestations of tension or fear during the conditioning With doses of 0 25–0 5 ml alcohol per rat (appr 1–2 mg/g), no effects were observed In experiments by the original WARNER technique FISCHER (1956) concluded that alcohol in doses of 1 5 or 3 g/kg did not affect the acquisition of the CAR in rats but did inhibit extinction

In Alko's laboratories, we are interested in objective methods of measuring the behavioural effects of alcohol, for we need such aids to studies of the physiology of intoxication and the development of tolerance to alcohol The experiments recorded here were undertaken in order to ascertain a) the effects of small and moderate doses of alcohol on the CAR of "non habituated" animals immediately after reaching a given level of performance, b) the effect of alcohol on the CAR of animals "habituated" to alcohol and c) the effect of alcohol on the CAR of animals that have undergone further training after learning the response

Experimental.

Apparatus A shuttle box of the type introduced by GELLHORN *et al* (1942) was used It measured 48 × 30 cm, with walls 45 cm high and was divided into two compartments by a partition with a 6 × 6 cm hole at the level of the grid floor The grids made of 3 mm brass rods spaced at 13 mm intervals and separately wired were suspended on sp[illegible] be separately charged The stimulator was a high

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after the animal had reached the required level of performance, previous experience of alcohol might also influence the results. For an evaluation of these possible effects, 30 rats were divided into three groups: I, 9 females and one male, II, 9 females and one male, and III, 6 females and 4 males. (Originally, all groups consisted of 5 females and 5 males. However, all animals that had not learned the response adequately after 450 trials were discarded, and this occurred mostly among the males, which were then replaced with females.) Training was as in expt. 2 except that the interval CS–US was 3 seconds. Reaction time and number of UARs were measured as in expt. 2.

Group I. Reaction time after administration of 1.5 ml/100 g of water was measured the day after the animals had reached the required level of performance. After this, 4 additional daily training sessions (30 trials) were given. The test session after administration of water was repeated, and on the final day of the experiment the effect of 3 mg/g of alcohol was measured.

Group II. Each animal received 3 mg/g of alcohol ten times at intervals of one day. After the animals had reached the required level of performance, the reaction time and number of UARs were measured on consecutive days after administering water and after 3 mg/g of alcohol. To make learning of the CAR and termination of the alcohol treatment coincide as closely as possible, alcohol was given a few times before training was begun. Even so, rapidly learning animals sometimes acquired the response before the end of the "habituation" period, and there was some delay before their performance could be measured. During training, the animals were given alcohol immediately after sessions and were rested the following day, in order to avoid any after-effects of alcohol on learning.

Group III served as a control for groups I and II. Training was as in group I, and the reaction time and number of UARs were measured as in group II.

Results.

Table 1 shows the results obtained in expt. 1. Both measures of performance, the reaction time and the number of UARs, appeared to be increased by alcohol. Only with the larger dose, however, was a significant effect obtained, that on reaction time barely exceeding the 5% confidence limit. Fasting had no apparent influence on the reaction to alcohol.

Table 1

Performance of the rats in [illegible]

mg alcohol/g	Reaction time sec.	P <	No. UAR	P <
0	1.78 (0.26)		0.8 (0.3)	
1	2.04 (0.63)	not sign.	2.3 (2.7)	not sign.
3	2.97 (0.80)	0.05	6.4 (6.2)	0.025
3 (fasted)	3.03 (2.10)	0.05	5.7 (4.9)	0.025

voltage (1100 V) low current (200–700 μA) device, giving approximately the same strength of shock independently of variations in the resistance of the animals A scrambling device continuously and irregularly changed the polarity of the rods The ringing of a bell served as the conditioning signal The shuttle box was covered with a transparent lid and placed in a quiet room, but it was not sound proofed

The reaction time was measured kymographically The switch for the bell also operated a magnet and pen, which recorded the beginning and end of the signal on the kymograph paper The drum of the kymograph was run at a speed of 15 mm/sec during trials and stopped between these In this way the reaction time was easily measured to within 1/10 of a second

General procedure All the rats used were from the laboratory stock Groups of 7–10 animals were housed in cages with grid floors and maintained on a standard diet and water *ad lib* Training was begun when the animals were 3 months old At the beginning of the first training session, the rats were allowed to explore the apparatus for 15 minutes Usually, they had access to food and water until the sessions began Alcohol was always given as a 20% (w/v) solution in tap water and administered by stomach tube The shock employed was generally 400 μA, although some large males were given up to 700 μA The bell signal (conditioned stimulus, CS) was given for either 3 or 5 seconds before charging the grid (unconditioned stimulus, US) The CS was stopped when the animal had run to the other compartment The usual interval between the beginnings of the trials was 30 seconds

The measures of performance used were a) reaction time = time taken to run into the other compartment after beginning the CS, b) number of unconditioned avoidance responses (UAR) = avoidance responses occurring only after the animals had received electric shocks

Experiment 1 This was a pilot experiment Eight female rats and one male were given 10 trials daily (interval CS–US 3 sec) until the animals made 9 correct responses during ten trials Subsequently, the reaction time and number of UARs were measured without pretreatment on two consecutive days After this, the effect of alcohol in 100 trials was measured according to the schedule 1 mg/g, 3 mg/g 1 mg/g, 3 mg/g The smaller dose of alcohol was administered 45 minutes before the sessions and the larger one 75 min before After an interval of 3–4 days, the animals were fasted overnight and re-tested with 3 mg/g alcohol, this treatment being repeated after one day of rest

Experiment 2 The pilot experiment suggested an improvement in the performance during the second session with the same treatment The procedure was therefore altered in this experiment, which was designed to show whether the dose-performance relationship is linear or nonlinear Twenty female rats were given daily sessions of 30 trials (interval CS–US 5 sec) After the animals had made 27 correct responses during one training session, they received 2 ml/100 g body weight of water on the following day, and the reaction time and number of UARs were measured after 75 min in 20 trials Immediately after this, 4 mg/g body weight of alcohol were administered and the animals were allowed to rest during the following day On the third [illegible] measured after [illegible] the fourth [illegible] tested for [illegible] Immediately before the reaction time was [illegible] ARVOLA, SAMMA-[illegible]) and WALLGREN [illegible] ARVOLA & SAMMALISTO 1960)

Experiment 3 The pilot experiment had indicated that, besides [illegible]

after the animal had reached the required level of performance, previous experience of alcohol might also influence the results For an evaluation of these possible effects, 30 rats were divided into three groups I, 9 females and one male, II, 9 females and one male, and III, 6 females and 4 males (Originally, all groups consisted of 5 females and 5 males However, all animals that had not learned the response adequately after 450 trials were discarded, and this occurred mostly among the males, which were then replaced with females) Training was as in expt 2 except that the interval CS-US was 3 seconds Reaction itme and number of UARs were measured as in expt 2.

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Table I

Performance of the rats in [illegible]

mg alcohol g	Reaction time sec.	P <	No UAR	P <
0	1 78 (0 26)		0 8 (0 3)	
1	2 04 (0 63)	not sign	2.3 (2 7)	not sign
3	2 97 (0 80)	0 05	6 4 (6 2)	0 025
3 (fasted)	3 03 (2 10)	0 05	5 7 (4 9)	0 025

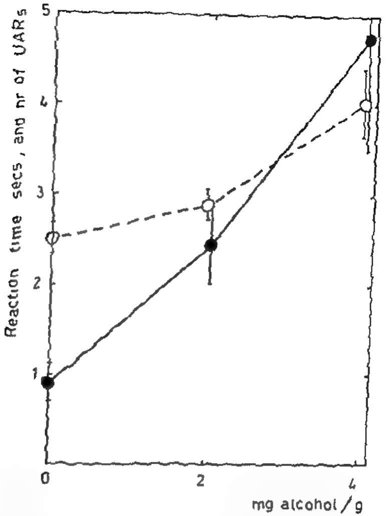


Fig 1 Performance of the rats in experiment 2 Reaction time (○ - - - - - ○) number of *unconditioned avoidance responses* (● —— ●) The points represent the average performance of 20 rats in 20 trials with each treatment Vertical bars indicate standard errors of the means

In fig 1, the performance of the rats in expt 2 is shown as a function of dosage of alcohol Alcohol clearly increased the reaction time and number of UARs (for statistical significance, see table 2) The regression of

Table 2

P values for significance of the effect of alcohol on various measures of performance in experiment 2 calculated by means of the t test for correlated measures

Measure	Alcohol 2 mg/g P <	Alcohol 4 mg/g P <	Difference 2 mg–4 mg P <
Reaction time	0 025	0 01	0 025
UARs	0 001	0 005	0 02
Tilted plane	0 001	0 001	0 005

reaction time on dosage deviates significantly from linearity (F for non-linear relationship = 13 749, P < 0 005) For the increase in number of UARs, the corresponding values are F = 7 903, P < 0 025 In the tilted plane test, 2 mg/g of alcohol reduced the angle of sliding by 11 1 ± 8 0% (average ± its standard deviation), and 4 mg/g by 19 4 ± 11 1% The level of significance is shown in table 2 This performance-dosage relationship does not deviate significantly from linearity Impairment of performance in the tilted plane test did not correlate with reaction time at the lower dosage level, but there was a significant correlation (r = +0 53, P < 0 025) after administration of 4 mg/g alcohol

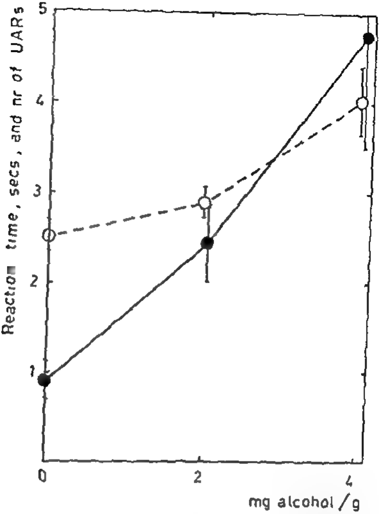
The main results of experiment 3 are summarized in table 3 In group I, continuation of training improved performance When first measured, the reaction time was 1 97 ± 0 22 sec and the number of UARs 1 7 ± 0 7, the reaction time being significantly (P < 0 05) longer than in the second session with water (table 3) However, continuation of training had no influence on the effect of alcohol, as was shown by the fact that the impairment of performance in groups I and III after administering alcohol was almost identical On the other hand, repeated administration of alcohol reduced its effect, since the performance of group II was not significantly impaired by the dose employed

Finally, the results show that reaction time was influenced by the time interval CS–US, which was 3 sec in expts 1 and 3, and 5 sec in experiment 2 The values for reaction time after administration of water in expts 1 and 3 have been pooled, and the significance of the difference of their mean from that of the value obtained in expt 2 has been calculated by means of Student's t test The means and their standard errors are 1 70 ± 0 27 sec (3 sec interval) and 2 51 ± 0 73 sec (5 sec interval) This difference is highly significant (P < 0 001) Variation is also signifi-

Table 3

Performance of the [illegible]

Group	Water	Alcohol	P <
I R t	1 63 (0 43)	2 26 (0 51)	0 005
UAR	1 0 (0 8)	4 1 (3 3)	0 01
II R t	1 57 (0 27)	1 79 (0 34)	not sign
UAR	1 1 (1 1)	1 9 (1 5)	not sign
III R t	1 81 (0 24)	2 29 (0 37)	0 01
UAR	1 1 (0 7)	4 2 (3 4)	0 05



(○- - - -○), number of
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Reaction time	0 025	0 01	0 025
UARs	0 001	0 005	0 02
Tilted plane	0 001	0 001	0 005

The results of all three experiments confirm earlier observations (HOLTEN & SONNE 1955, FISCHER 1956) that the CAR is relatively un affected by alcohol A major alteration in response is to be expected only in conjunction with gross motor disturbance, as evidenced by direct observation and the use of the tilted plane test after administering 4 mg/g of alcohol BATTIG & GRANDJEAN (1955) report a significant increase of reaction time in a group of 6–8 rats after administering as little as 1 6 mg/g of alcohol This may be due to the criterion employed, 50% correct responses, which was much less rigorous than the one used by us Also according to one report (WINTER & FLATAKER 1951) a positively reinforced (food reward) conditioned response is not easily affected by alcohol and is clearly impaired only when motor disturbance becomes visible

Fear is supposed to be an important factor in the CAR if this is so, the rather small effect of alcohol upon it appears to be somewhat at variance with results obtained in studies on the effect of alcohol in approach avoidance conflicts CONGER (according to MILLER & BARRY 1960) has produced good evidence for the view that alcohol reduces the avoidance tendencies without much influencing approach However, among possible explanations for this apparent discrepancy the mode of administering the fear inducing stimulus may be important In the shuttle box technique, electric shocks are usually avoided only when the animal escapes to the safe compartment, whereas the animals can more freely regulate the amount of current received when some kind of shock is given in approach-avoidance situations The shuttle box technique may thus induce a more deeply rooted fear reaction KORMAN (1960) has found that alcohol may reduce anxiety more effectively in low anxiety than in high anxiety" rats

It is a generally accepted view that recently acquired and less well mastered reactions are more easily disturbed by alcohol than earlier acquired and better controlled ones In our investigation, further training after learning to the level defined by the criterion adopted improved the performance of sober rats but did not influence the effect of alcohol (expt 3, table 3) One conceivable explanation is that owing to the rigid criterion of 27 correct responses out of 30 trials the rats learned the reaction so well that further training did not improve performance sufficiently [illegible]

[illegible] to alcohol after prolonged use of the drug is a well known phenomenon (cf JELLINEK 1960) This tolerance appears to be due both to psychomotor compensatory reactions and to a physiological change especially in the nervous system though the mechanism of the latter is unknown In experiment 3 administration of 3 mg/g of alcohol

cantly greater in the experiments with a 5 sec interval ($P < 0.001$) The effect of alcohol was not significantly affected by this change in experimental procedure

Discussion.

It seems appropriate to begin this section with a few notes about the technique used in this investigation MILLER & BARRY (1960) have pointed out certain difficulties involved in using the ordinary shuttle box Among these are development of conflict about re-entering the compartment where a shock was just received and the fact that animals given long sessions may loose the avoidance habit after having acquired it Such complications were encountered during the experiments reported here Preliminary tests indicated that they were aggravated if the rats had to jump over a hurdle in order to avoid the shocks Difficulties were also more frequently with males than with females, partly because many male rats developed freezing reactions These observations have some bearing on the design of experiments performed by the shuttle box technique One question is, whether one should use parallel control and experimental groups, or use the experimental subjects as their own controls as we have done The latter method may seem the less acceptable in studies on drug effects because of the possible loss of response and of the tendency, when reaction time is measured, to improvement in repeated sessions However, if parallel groups are used, between individual variation appears to be excessive The variation in the number of UARs is large, irrespective of the time interval CS–US In the experiments described here, variation in reaction time was reduced by diminishing the interval from 5 to 3 seconds, but it was still very large On the other hand, the direction of change was fairly consistent for each individual Also the results of repeated tests without drug administration, as in expt 1 and expt 3, group I, indicate that loss of response is not a serious drawback Use of the experimental subjects as their own controls thus seems to be the method of choice with this technique

Administration of alcohol to any fasted subjects results in a higher blood alcohol level than administration to satiated ones It was thus expected that the performance of the animals would be more affected after fasting (expt 1, table 1), That this was not so may have been due, first, to the fairly long interval between administration of alcohol and the test session, which perhaps minimized the effect of differing absorption rates, and secondly to increased tolerance More weight should perhaps be given to the latter factor (cf expt 3 effect of repeated administration of alcohol)

The results of all three experiments confirm earlier observations (HOLTEN & SONNE 1955, FISCHER 1956) that the CAR is relatively unaffected by alcohol A major alteration in response is to be expected only in conjunction with gross motor disturbance, as evidenced by direct observation and the use of the tilted plane test after administering 4 mg/g of alcohol BATTIG & GRANDJEAN (1955) report a significant increase of reaction time in a group of 6–8 rats after administering as little as 1 6 mg/g of alcohol This may be due to the criterion employed, 50% correct responses, which was much less rigorous than the one used by us Also according to one report, (WINTER & FLATAKER 1951) a positively reinforced (food reward) conditioned response is not easily affected by alcohol and is clearly impaired only when motor disturbance becomes visible

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The increase of tolerance to alcohol after prolonged use of the drug is a well known phenomenon (cf JELLINEK 1960) This tolerance appears to be due both to psychomotor compensatory reactions and to a physiological change, especially in the nervous system though the mechanism of the latter is unknown In experiment 3 administration of 3 mg/g of alcohol

every second day for 20 days evidently produced an increase in tolerance This was probably of physiological origin, since alcohol had not been given during the avoidance training sessions Recently, HOGANS, MORENO & BRODIE (1961) have reported development of behavioural tolerance to alcohol in monkeys when the drug has been administered once daily in a dose of 2 g/kg i v for as little as four consecutive days In Alko's laboratories, increased tolerance after a similar short period of habituation has been demonstrated by means of the tilted plane test (WALLGREN & LINDBOHM, in press)

Summary.

The effect of alcohol on a conditioned avoidance response (CAR) was studied in rats A two compartment shuttle box with a hole in the partition was used The conditioned signal was the ringing of a bell, the unconditioned stimulus was electric shocks from the grid floor given 3 or 5 sec. after the beginning of the signal A learning criterion of 27 correct responses out of 30 trials was used Reaction time and number of unconditioned avoidance responses (UARs = responses only after shock) were measured in test sessions of 20 trials

Reaction time and number of UARs increased after administration of alcohol However, the CAR was rather insensitive after administration of alcohol, major impairment was observed only in conjunction with overt signs of motor incoordination The smallest dose with which any significant changes were observed was 2 mg/g (orally administration) The performance dosage relationship was non-linear

Interindividual variation in reaction time was smaller with a 3 sec than with a 5 sec interval between signal and electric shock However, the variation is in any event so large that in studies on the effects of drugs use of the experimental subjects as their own controls seems preferable to the use of parallel control and experimental groups

Performance was slightly improved by continuing training after the animals had learnt the response This factor did not appear to change the influence of alcohol on performance Administration of 3 mg/g alcohol outside training sessions every other day for 20 days resulted in a diminution of the effect of alcohol, apparently due to increased physiological tolerance

Acknowledgement

The authors thank Prof Kai von Fieandt and Prof J M von Wright for helpful criticism of the manuscript, and Mrs Jean Margaret Perttunen for correcting the English

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Acta pharmacol et toxicol 1962, 19, 68–72

From the Research Laboratory of A/S H Lundbeck & Co

Hydroaminacrine I
Its Influence on the Interaction of Edrophonium and Suxamethonium

By

V. Dyrberg*, W. Hougs and S. H. Johansen*

(Received January 22 1962)

Hydroaminacrine (1,2,3,4 – tetrahydro – 9 – amino acridine) is a potent inhibitor of cholinesterase (SHAW & BENTLEY 1953, JENSEN-HOLM *et al*, *1961) It follows that the drug antagonizes the effects of* (+) tubocurarine and gallamonium, which both produce a non depolarizing block, and prolongs the effect of the depolarizing suxamethonium (GERSHON & SHAW 1958) It does not produce muscle paralysis by itself

It has been demonstrated on animals (ZAIMIS 1953) and man (CHURCHILL-DAVIDSON & RICHARDSON 1952, BRENNAN 1956) that prolonged exposure of the neuromuscular junction to depolarizing substances alters the characteristics of the block in such a manner that a non-depolarization block ensues This mode of action is described as "*dual block*" (ZAIMIS 1953)

Typical features of the dual block are

a) Initial relative insensitivity of the muscle towards the drug,
b) development of tachyphylaxis during the block,
c) that tetanic stimuli are poorly sustained and antagonize the block tetanic facilitation),
d) that the block is antagonized by cholinesterase inhibitors

In some species, such as dogs and rabbits, a dual block is rapidly induced, whereas in others, i e cat and man, it may take a considerable time to develop (ZAIMIS 1952) In man, CHURCHILL-DAVIDSON & CHRISTIE (1959) found evidence that the block of suxamethonium may change after doses of 500–1500 mg, given within one hour as a continuous infusion

The fact that hydroaminacrine prolongs and enhances the block of suxamethonium was first made use of clinically by CRANKSHAW (1960),

* Departments of Anaesthesia Copenhagen County Hospital in Gentofte Hellerup, Denmark

GORDH & WÅHLIN (1961) advocate this combination, because they feel that by reducing the amount of suxamethonium used they may protect their patients from the occurrence of dual block

The aim of our investigation was to test whether hydroaminacrine has any influence on the development of dual block The block was maintained continuously by suxamethonium, and the muscular reactions to repeated single doses of edrophonium were observed

Technique.

Since dual block develops speedily in rabbits, (ZAIMIS 1952), these animals were used Anaesthesia was by a combination of chloralose and urethane

The animals were artificially ventilated during the experiments through a tracheo- [illegible]

were cannulated one for continuous infusion the other for single injections

A continuous infusion of suxamethonium iodide (WHO,NFN) (= succinylcholine = curacit ®) was administered to 10 rabbits of which five were pretreated with hydroaminacrine 1 mg/kg After about 15 minutes of partial neuromuscular blockade the first dose of edrophonium (WHO NFN) (tensilon ®) 0 2 mg/kg was injected and subsequent doses were given at intervals of 15 minutes

Results.

During infusion of suxamethonium, edrophonium produced *three* different alterations in the neuromuscular block synergism, biphasic (brief antagonism succeeded by brief synergism) and antagonistic The *reactions* were mostly small and lasted for 2–3 minutes

The usual response to the first dose of edrophonium was one of synergism In two animals however, one of which was pretreated with hydroaminacrine the first dose gave an antagonistic response In the pretreated rabbits the biphasic response to edrophonium was observed *in* all five animals in three of them more than once, this reaction was only observed once in the untreated group Antagonism was eventually observed in all animals and the sequence of events in the two groups suggests that [illegible]

A biphasic response was taken as the first *sign* of antagonism

The amount of succinylcholine administered before antagonism developed, and the time when this *occurred*, are shown in table 1 On all

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ar ... sequence of events in the two groups suggests that the usual response to edrophonium in the given circumstances, is, first, synergism, then a biphasic response, especially when esterase activity is reduced, and finally antagonism

A biphasic response was taken as the first sign of antagonism

The amount of succinylcholine administered before antagonism developed, and the time when this occurred, are shown in table I On all

Table 1.

Amounts of suxamethonium (succinylcholine) infused at onset of antagonism to edrophonium, in five rabbits pretreated with hydroaminacrine and five controls

	Suxamethonium mg/kg	Duration minutes	mg/kg/min
	3 5	16	0 22
Hydroaminacrine 1 mg/kg	7 0	45	0 16
	7 1	45	0 16
	13 2	45	0 29
	7 0	45	0 16
Average	7 6	39 2	0 20
	20 9	35	0 59
	24 0	35	0 69
Controls	52 7	90	0 59
	22 4	17	1 32
	18 8	55	0 32
Average	27 8	46 4	0 70

occasions but one, the antagonism was observed within one hour, and the average times for the two groups were remarkably alike The amount of succinylcholine was reduced by 76% when the animals were pretreated with hydroaminacrine

Discussion.

Edrophonium has a bimodal type of action being partly an inhibitor of cholinesterase (BARLOW 1955), and partly exerting a direct acetylcholine mimetic effect at the muscle end-plate (RANDALL 1951) Investigations by HOUGS & JOHANSEN (1958) indicate that the cholinesterase inhibitory effect is the predominating one, although the "stimulating" properties are greater than those of neostigmine The two effects, however, both give rise to a pharmacological picture of increased acetylcholine concentration at the neuromuscular junction Edrophonium was chosen because its action is so brief that its accumulative effects may reasonably be disregarded

The antagonistic action of edrophonium in the presence of suxamethonium-induced neuromuscular blockade has been used previously (FOLDES 1959) for demonstrating a dual block Such an antagonistic action was observed in all our experiments, independently of pretreatment with hydroaminacrine The antagonism was in 8 out of 10 animals preceded by a synergism The time taken for the antagonism to develop varied in both groups, but there was no indication that hydroaminacrine had any influence on this The antagonism was observed on the average in less than an hour in both groups

The amount of succinylcholine administered before antagonism was observed was reduced by 76% in the group treated with hydroaminacrine, a figure close to that observed in man (BENVENISTE & DYRBERG 1962). This fact need not be interpreted as a sign that hydroaminacrine interferes in the development of dual block with succinylcholine. It is natural to expect a smaller dose to be needed when the enzymatic breakdown of this muscle relaxant is inhibited and the effect of a given dose consequently prolonged. After all, it must be the concentration of the curarizing agent at the neuromuscular junction that determines the degree of muscular paralysis. It is difficult to accept the suggestion of GORDH & WÅHLIN (1961) that, because smaller amounts of succinylcholine are needed for muscular paralysis in the presence of hydroaminacrine, this drug postpones the development of dual block.

In our experiments dual block, as indicated by the influence of edrophonium on suxamethonium induced muscular paralysis, seems to develop independently of the action of hydroaminacrine.

Summary.

The contractions of the anterior tibial muscle were recorded during continuous infusion of suxamethonium in rabbits. Single injections of edrophonium were given at regular intervals. A comparison was made between a control group of animals and a group treated with hydroaminacrine.

Antagonism between edrophonium and suxamethonium developed independently of the action of hydroaminacrine.

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Acta pharmacol et toxicol 1962, 19, 73–86

From the Biological Institute of the Carlsberg Foundation, Copenhagen

Variations in Non-Specific Activation of Fibrinolytic System in Sera of Different Species

By

E S Olesen

(Received February 10 1962)

It is possible to induce fibrinolytic activity in guinea pig serum by treatment with peptone or with various anionic and cationic agents (ASTRUP & OLESEN 1957, OLESEN 1957, 1959 a, b, c, 1960 a, b, c) Similar experiments have been carried out on samples of serum from other animal species, including man, and their results are reported here It was found that serum from different animal species differ in their ability to produce fibrinolytic activity under various influences The findings strengthen the view that a special activator inhibitor system is involved in certain paths of activating fibrinolysis in the blood This possibility has received little attention, although pertinent observations were reported by UNGAR & MIST (1949) and UNGAR, DAMGAARD & HUMMEL (1953)

Materials and Methods

Serum was obtained by spontaneous coagulation of fresh blood samples ...

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Th ... previous experiments on guinea pig serum and are briefly outlined below in the Experimental section Activity is expressed as the diameter product (mm²) of the lysed zone after 18 hours incubation of the fibrin plates at 37° (average of 3 determinations)

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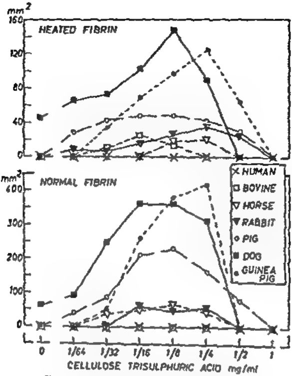


Fig 2 Fibrinolytic activity of serum from

Abscissa
Ordinate

2 *Activation by Peptone and by Taurocholate*

The observation by UNGAR & MIST (1949) that fibrinolytic activity could be produced in guinea-pig serum by peptone has been confirmed by us (OLESEN 1957) It was further demonstrated that a more effective 'purified peptone compound' could be prepared from commercial peptone by an acid ethanol precipitation (ASTRUP & OLESEN 1957) Peptone and

and taurocholate were therefore compared in different species (fig 3) Guinea pig serum, dog serum and pig serum yielded fibrinolytic activity with either compound, whereas human, bovine, horse and rabbit serum remained inactive In dog serum peptone produced higher activity than did taurocholate, whereas the reverse condition was found in guinea pig serum The activity produced in pig serum was only small

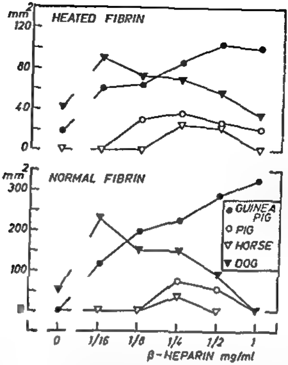


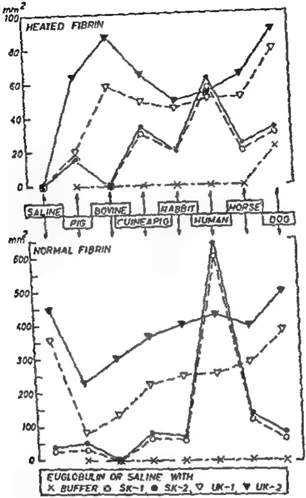
Fig 1 Fibrinolytic activity on normal and heated fibrin of serum from different species after isoelectric precipitation in the presence of various concentrations of β heparin (Bovine, human and rabbit serum also tested in this experiment produced no activity)

Abscissa mg of β heparin per ml of original serum volume (logarithmic scale)
Ordinate Fibrinolytic activity as diameter product (mm²) of lysed zone

Experimental.

1 *Activation by Acid Polysaccharides*

Isoelectric precipitation at pH 5 4 in the presence of various acid polysaccharides is an effective method for producing fibrinolytic activity in guinea-pig serum (Olesen 1959b) Among the compounds investigated, β-heparin was effective over a particularly broad range of concentrations, and cellulose trisulphuric acid produced higher activity than most other compounds The results obtained when either of these two compounds was applied to serum from different species are shown in fig 1 and fig 2 With β heparin the highest activities were formed in serum from guinea-pig and dog, smaller activities were produced in pig and horse serum, no demonstrable activity appeared in human, bovine and rabbit serum A similar order of succession was found for the activating effect of cellulose trisulphuric acid, but rabbit and bovine serum also became slightly fibrinolytic, though the activity of the bovine sample was only demonstrable on heated fibrin



(1953), streptokinase produced extremely high activity in human serum, especially when tested on normal fibrin

In each of the species, almost identical results were obtained with 250 and 500 units of streptokinase per ml of the test solution

Although streptokinase produced no demonstrable activation of the bovine euglobulin, the fact that it produced slight lysis on the normal (bovine) fibrin indicates that streptokinase has some activating effect in a more purified bovine system

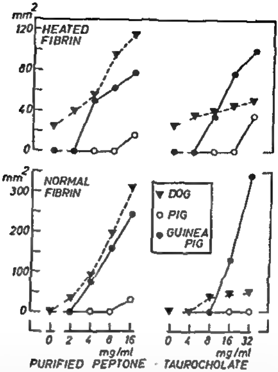


Fig 3 Fibrinolytic activity of serum after isoelectric precipitation with purified peptone or taurocholate (In this experiment bovine horse, human and rabbit serum produced no activity)
Abscissa mg of added compounds per ml of serum (logarithmic scale)
Ordinate Fibrinolytic activity

3 *Activation by Streptokinase*

The production of fibrinolytic activity by streptokinase seems to be different in type from the activation caused by peptone (Olesen 1959a)

In the experiments described below the euglobulin was prepared by isoelectric precipitation at pH 5 4 after dilution of the serum with 19 volumes of distilled water The precipitates were then dissolved in one volume of barbital buffer (pH 7 75, 0 05 M, containing 0 1 M-NaCl) Aliquots of these solutions were mixed with equal volumes of saline or saline containing either 500 or 1000 units per ml streptokinase (Varidase ® Lederle, kindly supplied by the manufacturer) Corresponding concentrations of streptokinase in saline were also applied as controls to the fibrin plates The results are shown in fig 4 Streptokinase alone produced a trace of lysis on normal fibrin, but no lysis on heated fibrin The euglobulin samples alone produced no lysis on any of the substrates, except the dog sample, which showed weak lysis on heated fibrin The lysis recorded on heated fibrin shows that addition of streptokinase produced activation in serum of all species except the bovine and possibly the dog, which showed only an insignificant rise in activity

As would be expected from the investigations by Mullertz & Lassen

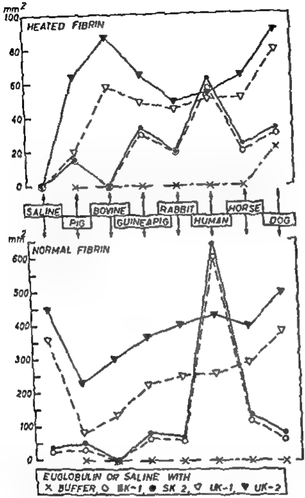


Fig 4 Fibrinolytic activity produced in different species by streptokinase or urokinase The test solutions contained saline or serum euglobulin, from the species indicated between the upper and lower parts of the figure and either buffer only or streptokinase 250 units (SK 1) or 500 units (SK 2) per ml or urokinase 0 25 mg (UK 1) or 0 5 mg (UK 2) per ml *Ordinate* Fibrinolytic activity

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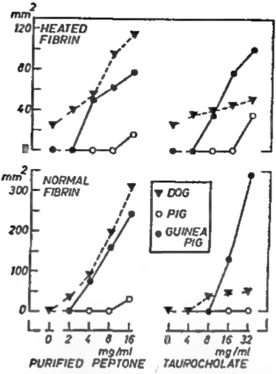

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quent spontaneous activation of plasminogen to plasmin (e g CHRISTENSEN & MACLEOD 1945)

Samples of serum used for these experiments were subjected to [illegible] uniform and simultaneous treatment with chloroform

[illegible]

an open Petri dish and aerated for 15 minutes by a current of air to remove residual chloroform After this treatment samples were applied to fibrin plates The remaining samples were placed in the refrigerator at 4° and assayed on subsequent days

As indicated in table 1, the dog serum showed fibrinolytic activity from the first day The guinea pig serum was active at and after the assay performed two days later the activities probably went on increasing for the next few days None of the other sera (human, horse, ox and pig) became active during the course of one week The activity produced in dog serum was about the highest ever observed in our experiments on the activation of fibrinolytic activity in serum from different species

Table 1

Fibrinolytic activity of chloroform treated dog serum and guinea pig serum [illegible] various intervals after treatment (The same treatment of bovine, human horse, and pig serum produced no activity)

Days after treatment		0	2	3	4	6
Dog serum	heated fibrin	105	330	306	470	484
	normal fibrin	0	585	1023	1450	>1500
Guinea pig serum	heated fibrin	[illegible]	103	107	121	100
	normal fibrin	0	0	90	113	110

6 *Inhibitory Effect of Serum*

The highly active chloroform treated dog serum, obtained in the experiment mentioned above, was used for comparison of the inhibitory effects produced by whole serum from different species These sera, diluted 1 10 or 1 25 with 0 9% NaCl, were added to equal volumes of the fibrinolytic dog serum The mixtures produced the fibrinolytic activities set out in fig 5 Bovine and guinea pig serum produced the [illegible] inhibition, [illegible] between this [illegible] the correspo[illegible] (as judged from fig 4) They are not strictly parallel, however, as euglobulin from the pig and whole serum from the guinea pig produced the strongest inhibition

4 *Activation by Urokinase*

Urokinase, a compound isolated from human urine, is an activator of plasminogen Aliquots of the euglobulins used for the above experiments with streptokinase were used in parallel experiments with urokinase (kindly supplied by the Leo Pharmaceutical Co, Copenhagen) The euglobulin solutions were mixed with equal volumes of saline containing per ml either 0 5 or 1 mg of urokinase (fig 4) and were compared with corresponding concentrations of urokinase alone in saline

Since urokinase alone produces lysis on normal fibrin, but no lysis on heated fibrin, its activating effect on the euglobulins has to be evaluated primarily from the results on heated fibrin, which represent the true protease activity of the test samples Urokinase produced protease activity of nearly the same magnitude in all seven species, though some what higher in bovine and dog serum than in the others In human serum the protease activity was almost the same as that produced by strepto kinase In the other species urokinase produced much higher protease activity than did streptokinase The plasminogen activation obtained with streptokinase was apparently far from complete The smaller concentra tion of urokinase usually produced less protease activity than the higher concentration, but only in pig ox and guinea-pig serum was the difference fairly pronounced

On normal fibrin, lysis represents the combined effects of the protease activated in the euglobulin and of the plasmin formed in the fibrin from its contents of plasminogen Only the mixture of urokinase with dog serum produced as high activity on normal fibrin as the same concentra tion of urokinase alone in saline The net effect of the euglobulin samples from other species was an inhibition of the urokinase effect This inhibi tion was most pronounced for the euglobulins from pig ox and guinea pig serum (which correlates the inadequate protease activation produced in these samples by the smaller amount of urokinase) The dog euglobulin appears to have the least inhibitory effect if it has any at all

These results with urokinase show that serum from each of the species here investigated contains a pro enzyme (presumably plasminogen) that is activated by urokinase to a protease (presumably plasmin), the euglo bulins produce varying degrees of inhibition

5 *Activation by Chloroform Treatment*

It is well established that treatment with chloroform can induce fibrino-lytic activity in serum, plasma or some plasma fractions This phenomenon is widely accepted as due to inactivation of inhibitory agents and subse

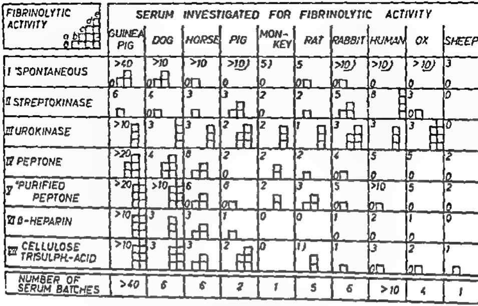

Fig 6 Magnitudes of fibrinolytic activity recorded for serum from different species after different procedures

I II III isoelectric precipitation in absence of added compounds II III streptokinase or urokinase added after precipitation

IV V VI VII isoelectrically precipitated in presence of the compo... nd ... ad ... d

th

or

purified peptone ... ell fore to ... 1

more effective T

lysis differed co

affected in the same way by these substances with the differences in degree mentioned above

These observations furnish circumstantial evidence for the concept that the activating effects of peptone (crude or purified) and of acid polysaccharides are the same in principle, (cf OLESEN 1959, b, c)

In fig 6 the species are arranged according to the level of fibrinolytic activity produced by precipitating their serum in the presence of these

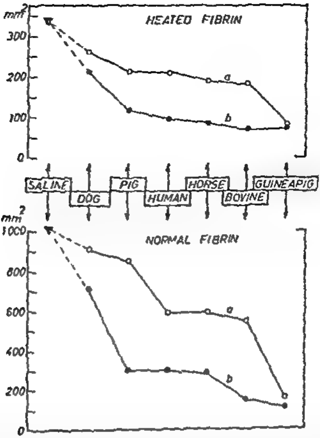

Fig 5 Fibrinolysis inhibiting effect of whole serum from different species The test solutions contain dog serum activated by chloroform treatment mixed with an equal volume of either saline (control) or untreated serum from the species indicated diluted (a) 1 25 or (b) 1 10

Ordinate Fibrinolytic activity

Discussion.

The results described above and additional comparative observations have been put together and summarized schematically in fig 6 and table 2 We can now describe some general trends in the formation of fibrinolytic activity in serum from different species in response to treatment with different compounds

A. Effect of Various Compounds

I Peptone and Acid Polysaccharides

Within each of the species investigated, there is a fair degree of correspondance of the effects produced by peptone, by 'purified peptone' and by the acid polysaccharides β-heparin and cellulose trisulphuric acid The 'purified peptone' was more effective than the crude peptone in inducing fibrinolytic activity; β-heparin produced almost the same effect as

II Cationic Compounds

Production of fibrinolytic activity in guinea pig serum by quaternary ammonium bases or by protamine sulphate has been described (OLESEN 1960b, c) The most effective of these compounds, cetyl pyridine chloride, produced fibrinolytic activity also on simple addition to guinea pig serum Direct addition to whole serum from other species gave positive results in dog and horse serum, but negative ones in human and bovine serum These findings are consistent with the possibility that this type of activation also is mediated through the same activator system as the activation induced by peptone and anionic compounds (cf OLESEN 1960b)

III 'Spontaneous Activation'

The simple isoelectric precipitation of serum at pH 5 3–5 4 in the absence of added compounds sometimes produced fibrinolytic activity, which has been called 'spontaneous activity' It appears from fig 6 that the occurrence of this phenomenon and the level of activity attained in different species showed a correlation with the fibrinolytic activity induced by peptone or acid polysaccharides This observation suggests that the formation of 'spontaneous' activity depends on the same plasminogen activator system as the 'peptone-activation', a concept in agreement with our previous results on guinea pig serum (OLESEN 1959a), corroborating the hypothesis proposed at that time, that the effect of peptone represents a modification and reinforcement of the mechanism responsible for formation of fibrinolytic activity by simple fractionation procedures

IV Chloroform

The activation of fibrinolytic activity in dog and guinea pig serum readily induced by chloroform treatment has been confirmed on other batches of serum from these two species The absent or occasional effect in some of the other species may indicate that the activation is caused by the same activator system' as the one mentioned above

V Urokinase

The effect of urokinase on serum from different species presents a different pattern from the activation by peptone and anionic compounds Activation of a serum protease (plasmin) is easily obtained in all the species by simple addition of urokinase to the euglobulin Even bovine

compounds Guinea-pig and dog serum have the highest potential activity At the other end of the scale are man, ox and sheep, and other species (horse, monkey, pig, rabbit, rat) in an intermediary position

Our studies on guinea-pig serum (OLESEN 1957 a, 1959 a, b) have shown that this type of activation involves the release of an agent that converts bovine plasminogen to plasmin and that this plasminogen activator is probably distinct from the activated serum protease as well as from the activator produced on adding streptokinase

Similarly we have observed that dog serum and horse serum, fibrinolytically active after precipitation in the presence of 'purified peptone', can activate bovine plasminogen Production of fibrinolytic activity in serum by peptone or by acid polysaccharides in general seems to be a phenomenon depending on the interaction of a special 'activator system' According to this view the activator system involved must be available in large amounts in dog and guinea pig serum, but hardly present in human, sheep and bovine serum

The distribution of this activating system in different animal species as outlined in table 2, is in accordance with various observations (1) Treatment of guinea pig serum with several other anionic compounds will readily produce fibrinolytic activity (OLESEN 1959 b, c) (2) Precipitation of serum from various species in the presence of another anionic compound, sodium taurocholate (cf OLESEN 1960a), produced fibrinolytic activity in guinea-pig, dog or pig serum, but no activity in bovine, horse, human or rabbit serum (3) Tauroglycocholate and α tocopherol phosphate, which produced fibrinolytic activity by simple addition to guinea pig whole serum or serum euglobulin (OLESEN 1960 a, 1959c), have been found to produce a similar effect in dog and pig serum but none in human or bovine serum This varied response, showing agreement in the individual species with the results obtained by the precipitation procedure, suggests that both activations depend on the same system

Table 2

Relative potency of component factors in the fibrinolytic systems of serums from different ... kinase and ... s observed ... nt

	Guinea pig	Dog	Pig	Horse	Human	Bovine
(1) *Plasminogen*	+++	++++	+++	+++	+++	++++
(2) Pro activator	+	(+)	+	+	++++	(+)
(3) Activator system	++++	++++	+++	++	(+)	0(+)
(4) Euglobulin inhibitors	++	0(+)	++++	+	+	+++
(5) Whole *serum inhibitors*	+++	+	++	++	++	+++

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serum, which is about the most resistent to activation by the other compounds, gives high activity with urokinase These observations indicate that a lack of plasminogen cannot be the limiting factor when defective activation is observed with some of the compounds mentioned

VI Streptokinase

Our results with streptokinase in different species confirm the well known difference between human serum and serum from other species The formation of extremely high fibrinolytic activity on normal fibrin is an indication of the potent plasminogen activator produced by addition of streptokinase to human serum Monkey serum (one sample, examined twice) was not activated by streptokinase In samples of dog serum and bovine serum we found either no unequivocal activation by streptokinase or occasionally slight activation In the other species (guinea pig, horse, rabbit, rat and pig) streptokinase produced slight to moderate activation

The complete lack of proportionality between the activations produced by streptokinase and by the other procedures under consideration in this paper (except urokinase activation) supports the view that streptokinase acts on a different precursor system (cf OLESEN 1959 b)

B 'Non-Specific Activation'

The experimental results presented in this paper show that sera from different species differ considerably in their responses to various forms of treatment aimed at producing fibrinolytic activity Special attention has been paid to those forms of activation produced by isoelectric precipitation, either 'spontaneously' (in the absence of added compounds) or in the presence of peptone or various anionic compounds Then the course of events, leading to the formation of fibrinolytic activity in the absence of any compounds having a direct effect on the fibrinolytic system (such as streptokinase, tissue activator or trypsin), seems to depend on interactions of a non-specific character with certain serum constituents Our results suggest that the formation of fibrinolytic activity induced by chloroform treatment belongs to this type of activation

The combined evidence from our experiments suggests that an 'activator system' in the serum is a prerequisite for 'non-specific activation' It also appears that the potency of this 'new activator system rather than the amount of plasminogen, is of importance for a serum's capacity for 'non specific activation', since plasminogen seems to be somewhat uniformly distributed in the species investigated The amount and character of inhibitory agents in serum probably also play a role On the other hand,

the potency of the 'pro-activator' system in serum, as indicated by the effect of streptokinase, has no relation to the 'non specific activation'

Since our experiments have been carried out on serum they may not be representative for the conditions prevailing in the blood of the animal species used Experiments with human plasma (OLESEN 1961) have shown that plasma differs from serum in that it regularly yields fibrinolytic activity when precipitated in the presence of 'purified peptone' or acid polysaccharides Human plasma must therefore be considered to possess an activator system more potent than that in serum

Summary.

The fibrinolytic systems in serum from different animal species have been compared by procedures involving isoelectric precipitation of serum in the presence of peptone or anionic polyelectrolytes, simple addition of streptokinase or urokinase to serum euglobulin and chloroform treatment of whole serum

Considerable differences between species were recorded Streptokinase and urokinase produced their own patterns of activating effect with no relation to the other types of activation The results suggest a relationship between the different non specific activating procedures investigated, as distinct from the activation produced by substances acting directly as kinases or activators of the fibrinolytic system, such as streptokinase and urokinase

Non specific activation' of fibrinolytic activity apparently depends on a special activator system This system seems to have high potential activity in guinea pig and dog serum, moderate potential activity in pig, horse and rabbit and low activity in human and bovine serum

Acknowledgements

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Central Depressant Activity of Some Thiaxanthene Derivatives

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(Received October 12, 1961)

In previous publications (PETERSEN *et al* 1958, MØLLER NIELSEN & NEUHOLD 1959) we have reported the central effects of some thiaxanthene derivatives, as well as detailed pharmacological studies of one of them, chlorprothixene (WHO)

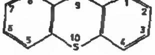
A close parallelism was demonstrated between the central depressant effect of thiaxanthenes and the corresponding phenothiazines Further, it was shown that the double bond between C-9 and the side chain was essential to any powerful central effect One compound, chlorprothixene, was separated as two isomers (cis and trans), the trans being about ten times more active than the cis form

Many thiaxanthenes have since then been synthesised and examined pharmacologically, in order further to study the structure activity relationship of thiaxanthenes compared with the corresponding phenothiazines, and also, when the isomers could be separated, to compare the biological activities of cis and trans forms This paper presents the results obtained with 66 derivatives in a few tests that are, in our exprience, well suited for ranking phenothiazines and thiaxanthenes as to central nervous depressant (neuroleptic) effect Detailed pharmacological studies of a selected group of compounds, which are being submitted for publication elsewhere, have largely confirmed the ranking established by means of these tests

Materials and Methods.

A Chemical

The compounds all contain the thirteen carbon and one sulphur ring structure



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The compounds were prepared as the most convenient water-soluble salts, (table 1, column 2) When no suitable salt was obtainable the solutions of the bases were prepared by adding hydrochloric acid The results shown in columns 5 to 9 are based on the substances as weighed

The assignment of cis or trans configuration to an isomer was based on measurement of refractive indices of the base in the amorphous state The details are being submitted for publications elsewhere

B Pharmacological

Acute toxicity was determined by intravenous injection into mice LD50 based on a minimum of 4 dose-levels (5–10 mice per group), was determined according to the method of MILLER & TAINTER (1944)

Motility Spontaneous motor activity of mice was determined in motility cages as previously described (KOPF & MØLLER NIELSEN 1959) DR50 represents the dose (mg/kg) that reduces motility to 50% of that of control mice

Potentiation of enhexymal anaesthesia in mice Groups of 10 mice were given 2 5 or 25 mg/kg i p of the test compound 30 minutes before i v injection of 50 mg/kg enhexymal sodium (hexobarbital (WHO), evipan ®) The potentiating factor was calculated as

$$\frac{\text{Sleeping time of test animals}}{\text{Sleeping time of control animals}}$$

Effect on body temperature of rats Rectal temperatures of rats were determined with a thermocouple readings being made every 15 minutes for 5½ hours Test compounds were injected i p at a standard dose of 5 mg/kg

Results.

Chemical and pharmacological results on 66 thiaxanthene derivatives and eight selected compounds in the phenothiazine series are shown in table 1 The compounds have been listed in groups according to the nature of the side chain at carbon-9 In the following discussion the compounds will be identified by their serial number in the table (column 1, above bar) rather than by their laboratory code number (column 1, below bar) The trans or cis isomer of any given compound is indicated in column 1 by the letter t or c, respectively, as suffix to the code number.

Table 1

1 Serial No / Code No	2 Substituent at 9		3 Other Substituent	4 Melting Point C	5 LD50 mg/kg i v	6 Mous i v DR50 mg/kg i p	7 Enhexymal Potentiating factor 2.5 mg/kg	8 Enhexymal Potentiating factor 25 mg/kg	9 Temperature Fall °C 5 mg/kg i p
1/N 762 t	$=CH\ CH_2\ N(CH_3)_2$	HCl	2 Cl	235–240	61	1.7	2	5	0.5
2/N 762 c		HCl	2 Cl	240–245	72	10	1.6	3	0
3/N 716	$=CH\ CH_2\ CH_2\text{–}N(CH_3)_2$	HCl		178–181	44	3.5	3	>7	2.3
4/N 7003 t		HBr	2 F	195–197	60	1.6	3	>9	2.7
5/N 7003 c		HBr	2 F	185–186	53	6	2	7	1.1
6/N 714 t		HCl	2 Cl	222–224	45	0.4	13	>43	4.6
7/N 714 c		HCl	2 Cl	212–214	35	4.7	2	6	0.8

Table 1 cont nued

1 Serial No / Code No	2 Substituent at 9	3 Other Sub-stituent	4 Melting Point °C	5 LD50 mg/kg i v	6 Moti lity DR50 mg/kg i p	7 Enhexymal Potentiating factor 2 5 mg/kg	8 Enhexymal Potentiating factor 25 mg/kg	9 Temper rature Fall °C 5 mg/kg i p
8/N 756 t	HCl	2 Br	222–224	79	0 2	4	21	1 7
9/N 756 c	HCl	2 Br	200–201	61	8	5	4	0 8
10/N 787 t	HCl	2 J	219–220	101	0 6	10	26	1 8
11/N 787 c	HCl	2 J	220–222	52	3 8	1 8	3	0 6
12/N 770	HCl	2 7 Cl	229–231	70	1 3	2	5	1 4
13/N 771	HCl	2 Cl, 7 Br	228–230	74	3 4	3	6	0 5
14/N 785	HCl	2,7 Br	213–216	131	14	3	4	1 0
15/N 796 t	HCl	2 CF_3	199–200	52	0 6	>8	>23	4 5
16/N 796 c	HCl	2 CF_3	218–220	33	6	3	6	1 0
17/N 741 t	HCl	2 OCH_3	172 173	52	0 6	7	~19	2 0

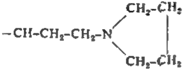
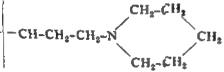
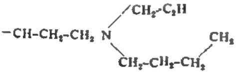
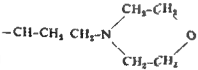
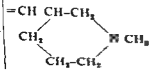
18/N 741 c		HCl	2 $COCH_3$	246–247	36	4 1	2	8	0 7
19/N 7008		HCl	4 Cl	204–206		6	1 5	5	0 6
20/N 797		HCl	4 J	220–222		6	3	9	0 4
21/N 794		HCl	1 CF_3	169–172	47	10	3	3	1 3
22/N 786	$=CH-CH_2-CH_2-N(CH_3)_2$	HCl	3 CF_3	197–199		11	2	3	0 6
23/N 788 A		HBr	3 CF_3	203–205		19	2	4	0 7
24/N 788 B									
25/N 781	$=CH-CH_2-CH_2-N(H)(CH_3)$	HCl	2 OCH_3	210–211	68	4 2	3	6	0 7
26/N 768 A	$=CH-CH_2-CH_2-N(C_2H_5)_2$	HCl	2 Cl	225–227	77	6	1 7	5	0 5
27/N 768 II		HBr	2 Cl	215–216	52	3 6	1 9	7	0 8
28/N 764 t	$=CH-CH_2-CH_2-N(CH_3)$ (ring)	HCl	2 Cl	212–213	67	13	1 1	4	0 6
29/N 764 c		HCl	2 Cl	187–188	72	6			0 6

Table 1 continued

1 Serial No / Code No	[illegible] Substituent at 9	3 Other Sub stituent	4 Melting Point °C	5 LD50 mg/kg i v	6 Moti lity DR50 mg/kg i p	7 Enhexymal Potentiating factor 2 5 mg/kg	8 25 mg/kg	9 Temper rature Fall °C 5 mg/kg i p
8/N 756 t	HCl	2 Br	222–224	79	0 2	4	21	1 7
9/N 756 c	HCl	2 Br	200–201	61	8	5	4	0 8
10/N 787 t	HCl	2 J	219–220	101	0 6	10	26	1 8
11/N 787 c	HCl	2 J	220–222	52	3 8	1 8	3	0 6
12/N 770	HCl	2 7 Cl	229 231	70	1 3	2	5	1 4
13/N 771	HCl	2 Cl, 7 Br	228–230	74	3 4	3	6	0 5
14/N 785	HCl	2,7 Br	213–216	131	14	3	4	1 0
15/N 796 t	HCl	2 CF_3	199 200	52	0 6	>8	>23	4 5
16/N 796 c	HCl	2 CF_3	218–220	33	6	3	6	1 0
17/N 741 t	HCl	[illegible] OCH_3	172 173	52	0 6	7	~19	2 0

39/N 727		HCl	2 OCH_3	[illegible]					
40/N 751	$=CH\,CH_2\,CH_2-N(CH_2CH_2)(CH_2-CH_2)NH$	2 HCl	2 Cl	150–170	82	14	2	6	0.6
41/N 748	$=CH\,CH_2\,CH_2-N(CH_2\,CH_2)(CH_2\,CH_2)N-CH_3$	2 HCl		265–275d	83	4	3	14	1.0
42/N 745		2 HCl	2 Cl	250–260d	105	1.2	3	6	1.2
43/N 775		2 HCl	3 OCH_3	233–236	74	4.4	1.3	9	0.8
44/N 7006	$=CH\,CH_2-N(CH_2-CH_2)(CH_2-CH_2)N-CH_2-CH_2-OH$	2 HCl	2 Cl	238–240	85	15	1	4	0.4
45/N 754	$=CH-CH_2-CH_2-N(CH_2-CH_2)(CH_2-CH_2)N-CH_2-CH_2-OH$	2 HCl		248–255	108	4.4	3	13	1.4
46/N 746		2 HCl	2 Cl	255–260	125	0.5	4	18	2.5
47/N 790		2 HCl	2 J	252–255	152	0.7	1.7	>13	2.7
48/N 772		2 HCl	2,7 Cl	260–270	53	2.1	1.9	8	1.0

Table 1 continued

1 Serial No / Code No	2 Substituent at 9		3 Other Substituent	4 Melting Point °C	5 LD50 mg/kg i v	6 Motility DR50 mg/kg i p	7 Enhexymal Potentiating factor 2 5 mg/kg	8 Enhexymal Potentiating factor 25 mg/kg	9 Temperature Fall °C 5 mg/kg i p
30/N 765 t		H_2SO_4	2 Cl	178–180	77	0 7	3	9	3 0
31/N 765 [illegible]		H_2SO_4	2 Cl	151–152	61	ca 4	7	10	1 1
32/N 753 t		H_2SO_4	2 Cl	203–208	74	1 4	>13	>60	2 8
33/N 753 c		H_2SO_4	2 Cl	190–192	91	12	4	12	0
34/N 7010 t		Base	2 Cl	oil	83	1 5	5	>25	1 7
35/N 7010 c		HBr	2 Cl	250–251	108	15	2	6	0 3
36/N 759		HCl	2 Cl	209–211		6 3	1 1	6	
37/N 724		HCl		257–259	100 i p	15	1 6	7	1 2
38/N 722		HCl	2 Cl	240 242		18	1 6	7	[illegible] 5

57/N 715	H, $CH_2\ CH\ CH_2$ / CH_3 $N\ CH_3$	Oxalate	2 OCH_3	190–192		16	1 6	4	0 7
58/N 728	$CH_3\ CH_3$	2 HCl		260	131 t p	21			0 4
59/N 711	H, $CH_2\ CH_2\ CH_2\ N(CH_2\ CH_2)(CH_2\ CH_2)N\ CH_3$	2 HCl	2 Cl	258 260	105	1 2	2	4	1 0
60/N 789	H, $CH_2\ CH_2\ CH_2\ N(CH_2\ CH_2)(CH_2\ CH_2)N\ CH_2\ CH_2\ OH$	2 HCl	2 Cl	238 243		6	4	9	1 1
61/N 792	H, $CH_2\ CH_2\ CH_2\ N(CH_2\ CH_2)(CH_2\ CH_2)N\ CH_2\ CH_2\ CH_2\ OH$	Base		155 156		17	1 4	3	0 2
62/N 705	OH, $CH_2\ CH_2\ CH_2\ N(CH_3)CH_3$	HCl	2 Cl	157 158		19	3	7	0 4
63/N 706		Base	2 OCH_3	120 121	60	10	1 6	15	0 9
64/N 7020		HCl		158 160		35	1 9	1 7	0 5
65/N 723	OH, $CH_2\ CH\ CH_2$					20	1 2	4	0

Table 1 continued

1 Serial No / Code No	2 Substituent at 9		3 Other Substituent	4 Melting Point °C	5 LD50 mg/kg i v	6 Motility DR50 mg/kg i p	7 Enhexymal Potentiating factor 2 5 mg/kg	8 Enhexymal Potentiating factor 25 mg/kg	9 Temperature Fall °C 5 mg/kg i p
49/N 769		2 HCl	2 Cl 7 Br	250–260	87	3 4	2	6	0 6
50/N 7009		2 HCl	2 CF_3	235–237	94	1 7	6	44	1 2
51/N 791		2 HCl	3 CF_3	140–180		3 7	2	4	0 7
52/N 773		2 HCl	2 OCH_3	242–245	102	1 7	3	9	2 1
53/N 761	$=CH-CH_2\ CH_2-N<{CH_2-CH_2 \atop CH_2\ CH_2}>N-CH_2-CH_2-OCOCH_3$	2 HCl	2 Cl	237–240	163	1 8	2	2	1 9
54/N 793	$-CH\ CH_2-CH_2-N<{CH_2-CH_2 \atop CH_2-CH_2}>N-CH_2-CH_2-CH_2\ OH$	2 HCl	2 Cl	245–250	118	2 1	2	8	1 1
55/N 710	$<{H \atop -CH_2-CH_2\ CH_2-N<{CH_3 \atop CH_3}}$	Base		oil		6	[illegible]	8	0 7
56/N 720		HBr	2 Cl	194–195	48	ca 7	3	>7	0 7

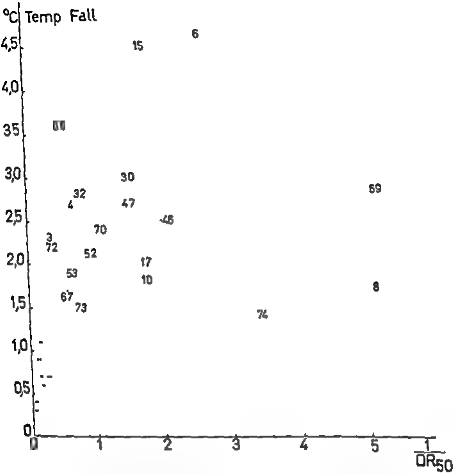
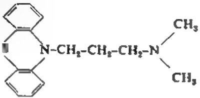
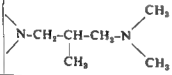
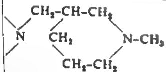
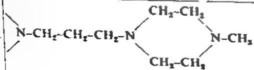
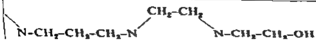


Fig 1 Relationship between motility reducing power and hypothermic activity of thiaxanthenes and some known phenothiazines

Abscissa Motility reducing power $\frac{1}{DR50}$

Ordinate Temperature fall after injection of 5 mg/kg test compound The most outstanding compounds are identified by their serial number in table 1 (3 = N 716 4 = N 7003 trans 6 714 trans 8 N 756 trans 10 = N 787 trans 15 = N 796 trans 17 N 741 trans 30 N 765 trans 32 — N 753 trans 46 = N 746 47 = N 790 52 = N 773 53 N 761 67 promazine 68 = chlorpromazine 69 = acepromazine 70 — levopromazine 72 prochlorperazine 73 — trifluoperazine 74 = perphenazine)

From the table it is evident that the thiaxanthenes represent a group of potent central depressant drugs, although considerable variation exists among the compounds With one exception (compound 60) the double bond between ring structure and side chain appears essential for any appreciable central effect Of the unsaturated compounds the most potent (numbers 6, 8, 10, 15, 17, 30, 46, 47) have a central depressant activity

1	2		3	4	5	6	7	8	9
erial No. / Code No.	Substituent at 9		Other Substituent	Melting Point °C	LD50 mg/kg i.v.	Motility DR50 mg/kg i.p.	Enhexymal Potentiating factor 2.5 mg/kg	Enhexymal Potentiating factor 25 mg/kg	Temperature Fall °C 5 mg/kg i.p.
7/Promazine		HCl			45	1.7	1.8	12	1.7
8/Chlorpromazine		HCl	2-Cl		54	2.4	5	26	3.6
69/Acepromazine		Maleate	2-COCH$_3$		65	0.2	16	>70	2.8
70/Levopromazine		HCl	2-OCH$_3$		75	1.0	11	>53	2.4
71/Mepazine		HCl			62	16	2	6	0.3
72/Prochlorperazine		CH$_3$SO$_3$H	2 Cl		101	3.8			2.2
73/Trifluoperazine		2 HCl	2-CF$_3$		82	1.5	4	>15	1.5
74/Perphena-		Base	2 Cl		44	0.3	1	1 6	1.4

Substitution in the 2 position generally lead to increased activity This was true for halogen substitution (compounds 4, 6, 8, 10 vs 3) Substitution with CF_3 or OCH_3 also appeared highly effective (compounds 15 and 17 vs 3, 50 and 52 vs 45) Among the 2 7 dihalogen substituted compounds the dichloro derivatives were the most active, though less active than the trans isomers of the 2 monohalogenated compounds (comp 12 vs 6)

On comparing the results obtained in the three tests a fairly good correlation appeared to exist between motility reducing power and temperature reduction In figure 1 maximal fall in body temperature after 5 mg/kg compound is plotted against the relative motility reducing power ($\frac{1}{DR50}$) The overall impression is that of an increased temperature effect with increasing motility reducing power, N 714 trans (chlorprothixene) being one of the most effective compounds in both tests Attention is called to the relatively weak hypothermic effect of perphenazine, acepromazine and N 756 trans (compounds 74 69 and 8), compared with their strong motility reducing power

In figure 2 motility reducing power is plotted against the barbiturate potentiating factor (enhexymal sodium) of a standard dose of 2 5 mg/kg of the test compound This diagram also appears to indicate a certain relationship between the two qualities perphenazine and N 756 trans (compounds 74 and 8) again representing striking exceptions with low potentiative effect compared with a strong motility reducing effect Of these two compounds perphenazine is even more outstanding, owing to its extremely low potentiating effect at the high dose of 25 mg/kg (table 1) It is noteworthy also that N 753 trans (compound 32) in spite of a comparatively low motility reducing power, is comparatively active in the enhexymal potentiating test

Summary.

Tests have been carried out on 66 thiaxanthene derivatives for motility reducing enhexymal (hexobarbital) potentiating and temperature reducing properties Like the phenothiazines the thiaxanthenes represent a group of very potent central depressant drugs There is a fairly close parallelism between the relative potencies of phenothiazines and those of the corresponding thiaxanthenes Three features appear essential for maximal central d…

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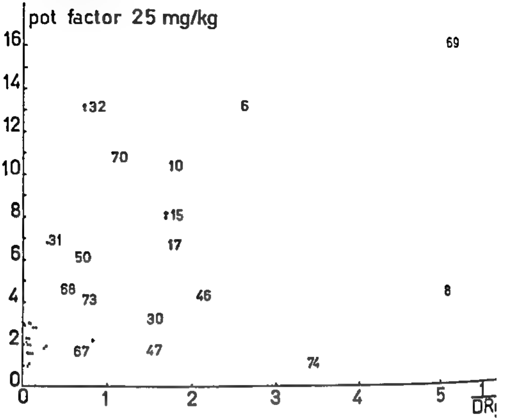

Fig 2 Relationship between motil ty reducing power and enhexymal potentiating fact
Abscissa Motility reducing power $-\frac{1}{DR50}$
Ord
N 787 trans 15 — N 796 trans 17 N 741 trans 30 N 765 trans 31 — N 765 (32 N 753 trans 46 N 746 47 — N 790 50 N 7009 67 — promazine 68 — chl promazine 69 acepromazine 70 — levopromazine 73 — trifluoperazine 74 — perpl nazine)

of the same order of magnitude as the most active phenothiazin acepromazine (69) and perphenazine (74) and several times greater th that of chlorpromazine (68)

Of the 2 substituted compounds 13 were separated as the two isome These compounds are listed between numbers 1 and 35 in the table 11 of these pairs of isomeric compounds identification of the cis a transform was successful For the remaining 2 pairs the isomers referred to as A or B Of the 2 substituted compounds with side cha containing more than one hetero atom only one isomer was obtaine which indicates that the other isomer either is unstable or was form in quantities too small to permit isolation Among the 11 compounds which identification of trans and cis isomers was successful, the tra form consistently appeared several times (5 to 40 times) more acti than the cis form (examples 6–7, 8–9, 10–11, 15–16 and 30–31)

Substitution in the 2 position generally lead to increased activity This was true for halogen substitution (compounds 4 6 8 10 vs 3) Substitution with CF_3 or OCH_3 also appeared highly effective (compounds 15 and 17 vs 3 50 and 52 vs 45) Among the 2 7-dihalogen substituted compounds the dichloro derivatives were the most active though less active than the trans isomers of the 2 monohalogenated compounds (comp 12 vs 6)

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Summary

Tests have been carried out on 66 thiaxanthene derivatives for motility reducing enhexymal (hexobarbital) potentiating and temperature reducing properties Like the phenothiazines the thiaxanthenes represent a group of very potent central depressant drugs There is a fairly close parallelism between the relative potencies of phenothiazines and those of the corresponding thiaxanthenes Three features appear essential for maximal central depressant activity 1) a double bond between carbon 9 and the side chain 2) substitution at carbon 2 and 3) trans configuration at the double bond

Acknowledgements.

The authors thank for their generous cooperation in supplying samples of known phenothiazines Ferrosan A/S, Copenhagen (perphenazine), AB Leo, Hälsingborg (levopromazine), Pharmacia A/S, Copenhagen (acepromazine) and Smith, Kline & French Laboratories, Philadelphia, Pa. (trifluoperazine).

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The Effects of Adrenalectomy and Administration of Desoxycorticosterone on Formation of Granulation Tissue

By

Ole Jørgensen

(Received January 23, 1962)

It is generally accepted that the adrenocortical hormones of the glucocorticoid type have an inhibitory effect on the process of healing The roles of desoxycorticosterone (desoxycortone (WHO, NFN), deoxycortone (BP)) and the other mineralocorticoids in this process are less universally agreed upon

In connection with the effect of the adrenal cortex on the process of healing it should be of interest to establish how healing proceeds after adrenalectomy, when the physiological secretion of adrenocortical hormones has been abolished

The available results of investigations into this problem seem to be contradictory *The work* recorded here has been carried out to throw light on the influence of adrenalectomy and administration of desoxycortone on the granulation tissue produced by the technique due to Rudas (1960)

Methods

Female rats weighing 80–100 g were used throughout

The adrenalectom [illegible] period The ope [illegible] control animals [illegible] operatively the [illegible] containing 1% [illegible] tomised animal [illegible] a few days

The desoxycortone treated animals were given daily *injections* of 0.5 mg desoxycortone acetate dissolved in 0 6 ml of arachis oil The *injections* were be[illegible] 5 da[illegible] before the exper[illegible]

Formation of granulation tissue The technique described by RUDAS (1960) was employed With the rat under ether anaesthesia a circular piece of skin was excised medially and distally from its back A plastic ring was inserted in the resulting gap The diameter of the ring was larger than the gap, so that it remained fixed there Further, the ring was provided with flanges to prevent its slipping All the rings had exactly the same inner diameter (20 mm), so that quantitative analyses of the granulation tissue formed in the rings were possible The animals were killed 8 days later and the rings were removed The granulation tissue formed was then easily removed by blunt dissection from the muscular fascia The tissue was weighed on a torsion balance immediately after excision and then dried

Determination of water content After weighing, the tissue was frozen at about −25° and dried in a desiccator over silica gel at a pressure of 1–3 mm Hg for 24 hours This procedure was repeated until the tissue reached constant weight The tissue was then defatted by shaking several times with a solvent in small perforated metal cases First light petroleum was used twice for 45 minutes each time and then anhydrous ether for 45 minutes at a time, until constant weight was reached (usually four times)

Hexosamine determination The hexosamine content was determined in dried defatted tissue by BLIX s (1948) modification of ELSON & MORGAN s method The tissue was hydrolysed for 15 hours with 2 N-HCl in sealed glass ampoules at 100° The hexosamine content was expressed as milligram hexosamine per 100 g dried defatted tissue

Collagen determination In the granulation tissue the content of collagen was determined as hydroxyproline multiplied by the factor 7 46 The dried defatted tissue was hydrolysed with 6 N-HCl for 24 hours at 100° in sealed glass ampoules The hydrolysate was filtered, and the amount of hydroxyproline was measured by MARTIN & AXELROD s (1955) modification of NEUMANN & LOGAN's method

Statistics For statistical purposes we used the t test

Results

In table 1 are recorded the weights of wet and dry defatted granulation tissue in the treated and the control groups It is clear that there was no difference between the groups

Table 2 shows the contents of hydroxyproline and collagen as well as the hexosamine concentration in the various animal groups The hydroxyproline content has been converted into collagen content by multiplying

Table 1

The weights of wet and dry defatted granulation tissue in the different groups
S e m , standard error of the mean, n, number of animals

	n	Wet Weight mg	Wet Weight s e m	Dry Weight mg	Dry Weight s e m
Control animals	14	460	25	73	3
Desoxycortone treated	15	478	26	76	3
Control animals	16	416	17	63	2
Adrenalectomy	14	436	25	65	3

Table 2

The contents of hydroxyproline and collagen and the hexosamine concentration in granulation tissues The values are expressed as mg [illegible] and mg per 100 g dry defatted tissue

	n	Hydroxy proline mg	Collagen g	Collagen s e m	n	Hexosamine mg	Hexosamine s e m
Control animals	14	1927	14 38	0 51	10	1069	29
Desoxycortone treated	16	1766	13 17	0 54	9	1063	23
Control animals	16	2179	16 26	0 43	8	1023	13
Adrenalectomy	14	2180	16 26	0 84	8	1038	14

by 7 46 The collagen is expressed as grams collagen per 100 g dry defatted tissue No changes in the parameters were found during the treatment given

Discussion

All reported histological examinations appear to have shown that injection of desoxycortone influences the process of wound healing PIRANI, STREPTO & SUTHERLAND (1951) administered the compound to guinea pigs and found that in laparotomy wounds the hormone stimulated proliferation of fibroblasts and delayed maturation of the collagenous fibrils was seen [illegible] the same time During the entire process of healing they observed an increased number of reticular fibrils and a reduced number of collagenous fibrils

TAUBENHAUS TAYLOR & MORTON (1952) submitted to histological analysis granulation tissue produced by injecting turpentine and showed that desoxycortone counteracted the inhibitory influence of cortisone Under the combined treatment with these two substances, abundant granulation tissue was formed but the tissue did not become normal, the fibroblasts lacking the regular orientation with cells lying parallel with the abscess cavity

In previous studies TAUBENHAUS & AMROMIN (1949, 1950), using the same analytical technique, found that treatment with desoxycortone given during the development of the turpentine abscesses had little effect on granulation tissue, whereas pretreatment with the substances resulted in formation of many multipolar fibroblasts, but with few collagenous fibrils

ROBERTSON & SANBORN (1958), on the other hand, by quantitative measurements of collagen in carrageenin provoked granulomas in guinea pigs, found that the amount of collagen was increased after treatment

with desoxycortone They state that the amount of granulation tissue seemed to be more abundant after treatment with desoxycortone, though they did not weigh the tissue formed However, VAN CAUWENBERGE & LECOMTE (1957), after having inserted a cotton plug, observed no change in its weight during treatment of the animals with desoxycortone, compared with the conditions in controls

In agreement with the latter result, our experiments gave no change in weight, either of the wet of the dry defatted tissue (table 1), and the collagen content was also found to remain unaltered after desoxycortone treatment

Histological studies by TAUBENHAUS & AMROMIN (1950) on the effect of adrenalectomy on granulation tissue in the walls of turpentine provoked abscesses revealed an inhibited fibroblast reaction, with small and scarce fibroblasts and slight collagen production The collagen lay arranged partly in fibres and partly in masses

In contrast with these results, ROBERTSON & SANBORN (1958) found a significant increase in collagen production by quantitative examination of carrageenin provoked granulomas in adrenalectomised guinea pigs PERNOKAS, EDWARDS & DUNPHY (1957) inserted ivalon sponges subcutaneously into rats and measured the amount of tissue formed and also determined the hydroxyproline content The values did not alter after adrenalectomy The results of these investigations accord completely with those of our work

Investigations into the hexosamine concentrations in granulation tissue during treatment with desoxycortone and after adrenalectomy have not been previously undertaken PIRANI, STREPTO & SUTHERLAND (1951) claim to have found an increased amount of intercellular substance in abdominal wounds under desoxycortone treatment, and they state that it showed increased staining In our work neither adrenalectomy nor desoxycortone treatment caused alterations in hexosamine concentration (table 2) However, this by no means precludes the occurrence of changes in the ground substance, since hexosamine concentration is no reliable indicator of changes in the connective tissue ground substance during wound healing (*vide* SCHMIDT 1960 and JACKSON, FLICKINGER & DUNPHY 1960)

The force required to disrupt laparotomy wounds in rats seems not to have changed significantly after adrenalectomy (CHASSIN *et al* 1954)

Considering the large doses of adrenocortical hormone required to inhibit the healing of wounds, cessation of the normal secretion by the adrenal glands seems unlikely to result in appreciably improved healing A comparison of our results and those of PERNOKAS, EDWARDS & DUNPHY with those of ROBERTSON & SANBORN makes it surprising that they found

increased collagen production after adrenalectomy It may, perhaps be explained by the fact that the latter workers had difficulty in keeping the animals alive without desoxycortone Administration of this hormone stimulated the collagen production in their experiments when given to non adrenalectomised animals

With stresses of different kinds, such as artificially provoked fractures, excision of large pieces of skin and infliction of burns, the tensile strength of 5 day old laparoatomy wounds was impaired, while at the same time the weight of the adrenal glands was significantly increased This fact argues in favour of the view that the adrenocortical secretion may sometimes be a factor of importance in the healing process (CHASSIN *et al* 1953) It is not certain, however, that the increased weight of the adrenal glands is the direct cause of the retarded healing The possibility must be considered that the injury causing the weight increase noted directly influenced the general state of the animals to such an extent that this alone interfered with healing The hypothesis of an inhibitory effect of a former stress on the process of healing is also in disagreement with the results of previous investigations by TAFFEL, DONOVAN & LAPINSKY (1951) These workers showed that a previous soft tissue lesion did not interfere with the healing of subsequent wounds in the rat stomach

The histological findings reported after adrenalectomy and administration of desoxycortone seem to be inconsistent with the biochemical demonstrations of an increased or unchanged content of hydroxyproline in granulation tissue during these treatments We cannot deny, however that the [illegible] collagen, manifesting themselves by reduced fibril formation, need not be inconsistent with the results of biochemical determination of the collagen content of granulation tissue, measured as hydroxyproline A possible inhibition of the extracellular maturation of collagen may result in a reduced number of collagenous fibrils without the hydroxyproline content being diminished, as the immature forms of the collagen contain hydroxyproline in the same amounts as the final protein As, however, wound strength after adrenalectomy is not impaired, inhibited fibril formation is not expected in this condition

To conclude we would say that, under the experimental conditions described neither adrenalectomy nor injection of desoxycortone seems to have influenced granulation tissue formation in general or more particularly collagen production

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Summary.

The effects of adrenalectomy and administration of desoxycortone on the formation of granulation tissue have been studied No signs have been found to indicate that these procedures influence granulation tissue formation, when assessed neither on the basis of the total amount of tissue formed or by the contents of collagen and hexosamine

Acknowledgement

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Toxin of the Weeverfish *(Trachinus Draco)* Experimental Studies on Animals*)

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Einar Skeie

(Received February 21 1962)

Ever since suspicion was aroused about the venomous nature of the weeverfish (*Trachinus draco*), reports have appeared at intervals on the effects of its poison on various animals The earlier work falls into two main groups experiments carried out by administering the venom to animals, and then describing the effect by simple observation, and experiments in which some form of apparatus has also been used to record the effect of the toxin

The first mentioned group is by far the larger SCHMIDT (1874) used frogs and rabbits and carried out implantation of weeverfish glandular tissue besides stinging the animals with the spines of the fish However, he used mainly fish that had been stored in spirit, and no effect could be observed Since then in the main GRESSIN (1884), BOTTARD (1889), PHISALIX (1899) and BRIOT (1902a) have carried out studies with weever toxin on smaller animals The results of these experiments differ greatly, probably owing to the heterogeneity of the toxic material available Only BRIOT seems to have worked with fairly strong toxin He carried out his experiments on various animals and gives paralysis of the respiratory muscles as the cause of death

The second group comprises only few studies EVANS (1907) published a study in which he recorded the blood pressure and respiration of a cat after injecting a lethal dose of weever venom He found that the blood pressure fell considerably immediately after the [illegible]
was
the

*) Part of the work was carried out at the Pharmacological Department of Messrs H Lundbeck & Co Financial assistance has been granted by the P Carl Petersen s Foundation and the Danish State Research Foundation (Statens almindelige Videnskabsfond)

performed extensive experiments on the haemolytic effect of weeverfish toxin and were able to show that it produced *in vitro* haemolysis of the blood corpuscles of various animals DE MARCO (1936) observed an increase in potassium permeability in the central nervous system of *Bufo viridis* after injection of weeverfish venom and was of the opinion that this poison enhanced the irritability of elements of the central nervous system In 1937 he observed increased reflex irritability with cramp and paralysis in frogs injected with weeverfish toxin In a study dating from 1938 he found more rapid exhaustion of isolated frog gastrochnemius in the effect of weeverfish venom On the basis of guinea pig experiments MARETIC (1957) assumed that weeverfish toxin contains hyaluronidase The most recent investigations in this group have been carried out by RUSSELL & EMERY (1960) They performed cardiovascular experiments on cats and observed among other effects a rapid fall in blood pressure simultaneously with an increase in venous blood pressure after injection of a lethal dose They also found electrocardiographic and electroencephalographic changes but no effect on the neuromuscular junction in nerve diaphragm preparations from guinea pigs

All the studies reported hitherto suffer from the disadvantage that no stable preparation of well defined toxicity has been available It has therefore been impossible to carry out the experiments in a quantitatively satisfactory way, because of this the results have been rather haphazard As mentioned previously (SKEIE 1962a) we have been successful in finding a method for extracting and storing weeverfish toxin Several experiments have been carried out with this toxin on various animals and some of these will be reported here

Experimental Methods

The same batch of weeverfish toxin (FJ A) from *Trachinus draco* was used in all the experiments It contained 640 lethal doses per ml*)

White mice weighing 16 to 18 g were injected with various concentrations of the toxin either subcutaneously on the back intraperitoneally or intravenously into a tail vein Guinea pigs also were injected with various concentrations of the toxin intravenously into an ear vein The effect of the toxin on the animals was observed and autopsy was performed on large numbers sometimes with subsequent microscopical examination of the organs

Blood pressure and respiration were measured and electrocardiograms were taken before during and after injecting different concentrations of the toxin into rabbits and cats For the latter the experimental procedures and technique used were as described below

*) Groups of white mice weighing 16–18 g were given the toxin intravenously in doses Th[illegible] ly called DML/100 or [illegible] in a group Generally

Rabbits Anaesthetic Urethane 25% 5 ml (1 25 g)/kg intravenously The structures of the neck were exposed Tracheotomy was performed and a cannula inserted and connected directly by tubing to a tambour for recording the respiration A recorder was also attached to register every 1 100 respiration excursion By means of a supplementary tube with a Jaquet valve with low dead space, the cannula in the trachea stayed affixed [illegible] a Gildemeister gas meter for measuring the ventilation A recorder registered a mark for every litre of air expired The carotid artery was connected to a mercury manometer A cannula was inserted into the left jugular vein for injections during the experiment

Cat Anaesthetic 1% chloralose and 25% urethane in the ratio of 5 2 Dose 6 ml/kg (426 mg urethane and 42 6 mg chloralose/kg) One third of this was injected intraperitoneally and two-thirds intramuscularly Cannulas were inserted into the femoral artery to measure the blood pressure and into the femoral vein for injection Otherwise the experiment was carried out in the same way as those with rabbits All the animals were previously given 500 units heparin (Leo)/kg

An attempt was made to examine the effect of weever toxin on the *peripheral circulation* by means of a preparation of the hind quarters of a *rat* by the technique described by Burn (1952), with some modifications The experimental procedure was briefly as described below The abdominal cavity of an ether anaesthetized rat was opened The rectum was divided off with ligatures The superior and inferior mesenteric arteries were tied, after which the intestines were removed from below in an upwards direction A cannula was fixed in the abdominal aorta, and the body of the rat was severed proximate to the cannulation The vascular system was perfused through the cannula with a modified Ringer solution heated to 37°C under 300 mm water pressure By means of a side tube the toxin was introduced into the perfusion fluid The fluid issuing from the venous side was collected on the slanting pad on which the animal was lying The fluid was allowed to drip past a photo cell, from which the impulses were transferred to an ordinate recorder

In agreement with the investigations mentioned above (Briot 1902*b*, Evans 1907, 1910) we were able to demonstrate a *haemolytic effect* of weever toxin on the blood corpuscles of various animals *in vitro* In order to judge whether and to what extent this effect is of significance in the syndrome of weever intoxication, another experiment was performed —

Various concentration[illegible]
about 2 kg Blood sampl[illegible]
injections Control anim[illegible]
toxin The blood [illegible]
examine[illegible]
as a perc[illegible]
means o[illegible]

Results

Direct Observations on Mice and Guinea-pigs

After intravenous injection of several lethal doses into white mice, death occurred instantaneously There was just time to observe a rapid spasm in the extremities and body during the injection, but after that the respiration and heart action ceased at once

Fig 1 Ear necrosis in white mouse 8 days after injection of toxin the right ear is about to fall off

After injection of 1 DML of the toxin strong motor disturbances could be seen immediately The mice began to run round in circles and after a few seconds fell on their sides or backs in opisthotonic postures Tonic and clonic spasms developed in the extremities Respiration became gasping and slower Sometimes a frothy discharge could be seen from the nose and mouth The animals usually died in a few seconds to minutes The reactions observed in mice were also seen in guinea pigs

An immediate *reaction sometimes also succeeded injection of* sub lethal doses The mice became dyspnoeic and quiet but recovered quickly The periphery of the thin, transparent ears of the mice rapidly became white, and no blood vessels could be seen because of the vascular contraction In animals observed over longer periods, it was noted that already 24 hours after the injection the periphery of the ears and the tip of the tail had become darker in colour The colour became intensified during the subsequent 24 hours, and *ischaemic necrosis of the ears and tip of the tail* developed In four to five days the greater part of the ears became ab solutely desiccated, stiff and black There was a clear zone of demarca tion between the fresh and the necrotic tissue, and finally the ears fell off (figs 1 and 2) The same applied to the tip of the tail

Four to five days after the injection the hair of the mice injected with sub lethal doses began to fall out in a peculiar way, beginning in the form

Fig 2 Both ears have fallen off as the result of necrosis Photograph taken 8 days after injection of toxin

of a cross on the back and spreading out to the sides Sometimes large flakes of skin covered with hair fell off at a later stage (fig 3) These ischaemic consequences have briefly been described previously (SKEIE 1962b)

The tolerance for weever toxin differs from one animal species to the other and is greatly dependent on the mode of administration Comparative experiments with guinea pigs, rabbits and mice showed that after intravenous injection 1 DML for guinea pigs weighing 700 g was equal to 4 DML for mice weighing 16 to 18 g and that 1 DML for rabbits weighing 2000 g was equal to 8 DML for mice This shows that, on a weight basis, guinea pigs are about 10 times and rabbits about 25 times more sensitive to the toxin than mice

Four times more toxin is needed to kill white mice by intraperitoneal than by intravenous injection and sixteen times more by subcutaneous injection

The symptoms also differed with the method of injection After subcutaneous and intraperitoneal injection, except after extremely large doses, no spasms were seen like those generally observed after intravenous injection The animals became dyspnoeic, quiet and immobile They be-

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Four to five days after the injection, the hair of the mice injected with sub lethal doses began to fall out in a peculiar way, beginning in the form

Fig 4 Record of respiration and blood pressure after injection of weever toxin into a rabbit
A Respiration B Ventilation in litres C Respiration frequency 1 100 D Arterial blood pressure E Injection scale F Time scale in minutes The small letters on curve D indicate at what stages parts of electrocardiograms are shown
I II and III on injection scale E indicate injections of 2, 4 and 40 mouse DML respectively

and ventilation, although falling, remained somewhat higher than normal Doses of 2 and 4 DML were administered 45 and 58 minutes after the first injection Both had a rapid but transient effect on the blood pressure Another 4 DML were given 66 minutes after the first injection (point II) This caused a sharp fall in blood pressure for half a minute and then a slight rise and a subsequent fall to 30 mm Hg Respiration frequency was increased, this time to 130/min, and increased depth of respiration and increased ventilation to 2900 ml/min were observed In the course of about 60 minutes the values were almost normal again At this stage (point III) 2 hours 50 minutes after the first injection, a dose known to be lethal for the rabbit (40 mouse DML) was given In the course of a few seconds the blood pressure fell sharply There was apnoea for about a minute Respiration then began again suddenly, became deep and gasping and then ceased completely after 1½ minutes At the same time the blood pressure fell to nil, and the heart action ceased immediately after the respiration stopped

Fig 5 shows part of an *electrocardiogram* lead II taken during the experiment The small letters refer to the corresponding letters on the blood pressure curve At point b there was slight sinus arrhythmia and

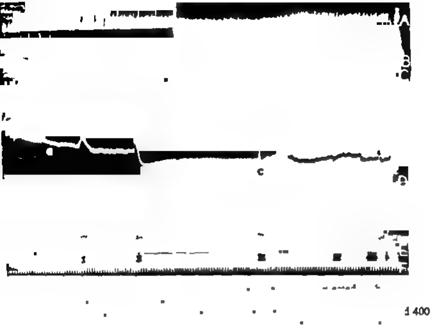
Fig 3 Necrosis of ears and tip of tail and loss of hair
Photograph taken 10 days after injection of toxin

came absolutely stiff in the "normal anatomic" position, gradually became iller and died imperceptibly without premortal excitation

After subcutaneous and intramuscular injections large areas of necrosis appeared at the site of injection However these were not seen after repeated injections, which could be interpreted as indicating the development of immunity

Recorded Observations

Fig 4 shows the record of respiration and arterial blood pressure in a *rabbit* under the influence of various concentrations of weever toxin expressed as mouse DML/100 The initial values were, for arterial pressure 90 mm Hg, respiration frequency about 100/min and ventilation about 2000 ml/min There was no effect on respiration or blood pressure after injection of 1 DML of toxin Three minutes later 2 DML were administered, again without effect Seven minutes after the first injection (point I on injection scale E) 2 DML were given After 30 seconds there was a sharp fall in blood pressure from 90 to 60 mm Hg in the course of one minute At the same time the respiration frequency increased to 120/min and the ventilation to 2500/min The blood pressure became almost normal again after about 20 to 25 minutes, whereas the respiration frequency



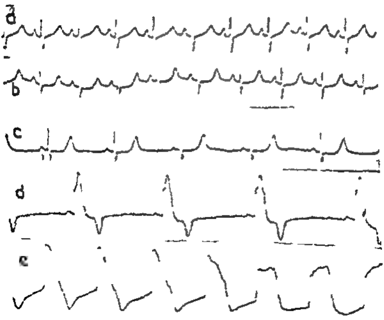


Fig 7 Section of electrocardiogram lead II
The small letters refer to the corresponding letters on curve D in fig 6

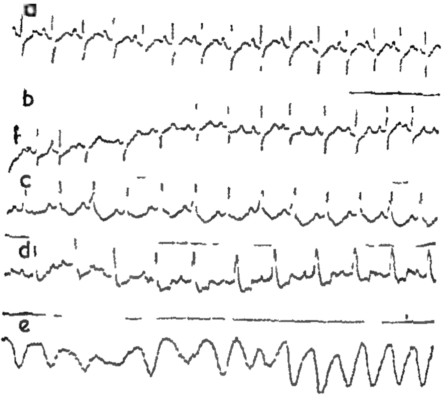


Fig 5 Section of electrocardiogram lead II
The small letters refer to the corresponding letters on curve D in fig 4

at point c a slightly broader initial complex with severe depression of the ST wave The same changes can be seen at point d Ventricular flutters occurred just before death, corresponding to point e

Fig 6 shows a similar experiment carried out on a *cat* Respiration was affected in the same way as in the rabbit, with increased respiration frequency and depth after administering the toxin, but the effect on the arterial pressure was somewhat different At the beginning of the experiment the arterial blood pressure was 120 mm Hg with a slightly decreasing tendency At stage I on injection scale E a dose of 2 DML was given This resulted in a gradual increase in blood pressure of 20 mm Hg during two minutes Then came a somewhat slow decrease back to the slightly decreasing basic level At 22 minutes later (point II) 4 DML were administered This resulted in a steep rise in the blood pressure in the course of 10 seconds and then a slow decrease to 60 mm Hg i e 20 mm below the pre-injection level, where it remained A dose of 2 8 ml weever antitoxic rabbit serum was given 70 minutes after the first injection (point III) Immediately after the injection a rapid swing in the blood pressure occurred, with a subsequent transient slight elevation lasting about 15 minutes Doses of 4, 32 and 128 DML were administered 25, 39 and 45 minutes, respectively,

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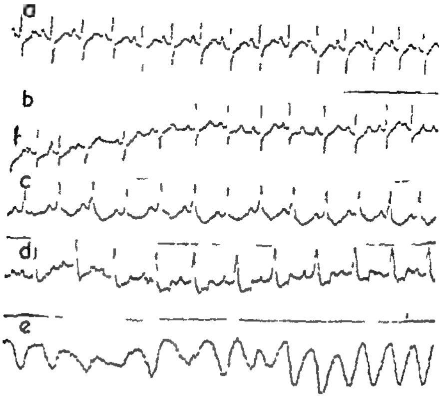
The small letters refer to the corresponding letters on curve D in fig 6



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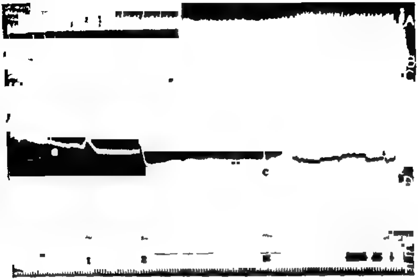

Fig ı

I II IV V
mouse DML ı

1 400

Fig 7 Section of electrocardiogram lead II
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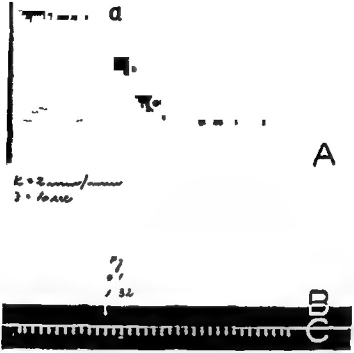

Fig 8 Ordinate curve from perfusion experiment on the hind quarters of a rat

A Basic level of ordinate curve B Injection scale C Time scale in minutes I Rising time of ordinate recorder k Drum speed

a Shows the sudden drop in the curve after injection of 2 DML

b to [illegible] Shows the more gradual slope During this period the hind quarters of the rat became tense and oedematous

after the serum injection (points IV, V and VI) These had only a slight effect on blood pressure and respiration About 400 DML were given 50 minutes after the serum injection (point VII) A marked rise in blood pressure occurred immediately and then a rapid fall to nil In the cat apnoea of about one minute's duration also occurred after the lethal dose had been given, with a few extremely deep respirations just before death

Fig 7 shows part of the *electrocardiogram* taken during the experiment As in the previous experiment, the small letters refer to the corresponding letters on the blood pressure curve The first important changes occurred after the weever antitoxic serum injection was given (point III) when considerable bradycardia was seen (c) Just before death (d) the ST wave was depressed, the T wave was negative, and finally (e) there was ventricular flutter

Fig 8 shows an ordinate curve drawn during *perfusion of the hind quarters of a rat* At constant perfusion pressure equilibrium is attained The

beginning of the curve is a straight line up to point a A few seconds after weever toxin has been introduced into the system a vertical fall of the curve occurs from point a to point b During the next five minutes there is a more gradual decrease up to point c During this period the hind part of the body of the rat becomes greatly swollen and is tense and oedematous An equilibrium is then established, and the curve again takes a horizontal course

The *haemolysis* experiments showed that in the blood samples taken up to two minutes after the injection of 1 DML (8 mouse DML) to a rabbit weighing 2000 [redacted] there was about 20% haemolysis After injection of a sub lethal dose (4 mouse DML) there was about 3% haemolysis The same results were found in blood samples taken 2 hours later In the control animals no haemolysis occurred

We have carried out *autopsies* on many animals – mice, guinea pigs and rabbits The pathological change most frequently found was pronounced stasis, primarily in the lungs and liver Frequently a frothy discharge was seen in the respiratory system In one rabbit the secretion was bloody, with severe haemorrhagic discoloration sub-pleurally The heart was always full of blood on the right side The left ventricle contained no blood Sometimes the heart was dilated Microscopy of the organs showed no pathological changes, except for the stasis However, this could hardly be expected, in view of the short period of illness, but the mice with severe ischaemic ear changes also were found to be without pathological processes in the other organs

Discussion

Animal poisons are generally complex containing several components that often have different effects It would be natural to assume that the same applies to weeverfish toxin

The most obvious and characteristic macroscopic effects of weeverfish toxin are the necrosis of the ears and tip of the tail and the peculiar loss of hair in mice These processes are undoubtedly caused by strong vascular contraction with consequent ischaemia and malnutrition of the tissues

This vascular effect has been clearly demonstrated experimentally in the perfusion experiment with the rat hind leg preparation The slanting section b to c on the curve in Fig 8, where the preparation becomes oedematous is presumably caused by the high content of hyaluronidase*)

*) The *hyaluronidase concentration* in the batch of weever toxin (Fj A) used in the above experiments was turbidimetrically and viscosimetrically found to be 38 600 I U /ml The determination was made by the courtesy of Messrs Lundbeck & Co and Messrs Leo Helsingborg

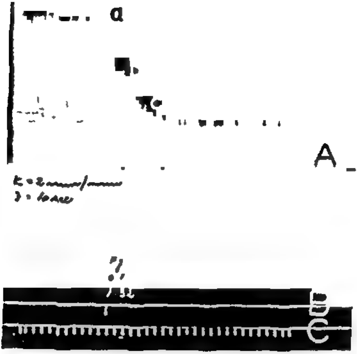

Fig 8 Ordinate curve from perfusion experiment on the hind quarters of a rat

A Basic level of ordinate curve, B Injection scale, C Time scale in minutes I Rising time of ordinate recorder K Drum speed

a Shows the sudden drop in the curve after injection of 2 DML,

b to c Shows the more gradual slope During this period the hind quarters of the rat became tense and oedematous

after the serum injection (points IV, V and VI) These had only a slight effect on blood pressure and respiration About 400 *DML* were given 50 minutes after the serum injection (point VII) A marked rise in blood pressure occurred immediately and then a rapid fall to nil In the cat apnoea of about one minute's duration also occurred after the lethal dose had been given, with a few extremely deep respirations just before death

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As would be expected, the toxicity was greatest after intravenous administration presumably because of the rapidly resulting vascular contraction In white mice 1 DML intravenously is equivalent to 4 DML intraperitoneally and 16 DML subcutaneously With the two last routes of injection, the sharpness of the effect is lessened because of the spreading phenomenon and the more gradual absorption

It will be seen from the experiment on the cat mentioned previously that, whereas early in the experiment administration of only 2 DML had [illegible] marked effect on the blood pressure and respiration, such reaction was almost absent, even after injection of a total of about 150 DML, if a specific antiserum had been given beforehand Thus the most important effect of weeverfish venom can be neutralized by specific antibodies

Summary

A brief survey is made of the experimental studies with weeverfish toxin previously carried out on animals Several of the author's experiments on various species of animals are described and particular reference is made to the vascular contracting effect of the toxin as being of fundamental significance This effect is demonstrated experimentally by perfusion experiments with a rat hind leg preparation The influence of weever venom on blood pressure and respiration has been measured on rabbits and cats *In vivo* experiments on rabbits have shown a pronounced rapid haemolytic effect of weever toxin The primary cause of death is considered to be heart failure

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De Marco [illegible] Effetti del veleno di Trachinus e d [illegible]

De [illegible]

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In our opinion the component responsible for the vascular contraction is of fundamental importance in the effect of weeverfish toxin and may help to explain the origin of the most important manifestations of the intoxication, i e the severe necrosis at the site of injection and the local necrosis that also occurs frequently in human subjects after a weeverfish sting (SKEIE 1962b) However, this effect is even more general It would seem natural to assume that the opisthotonic position with tonic and clonic spasms, quite characteristic for the animals that die after the administration of weever toxin, could be caused by brain ischaemia due to vascular contraction Further, the ischaemic myocardial changes seen on the electrocardiogram after injection of weever toxin into cats and rabbits may also have a similar origin

Comparison of these findings with the clinical observations makes it likely that the intense local pain and stenocardia in humans after a weeverfish sting are caused by severe vascular spasms with ischaemia

There is much evidence to indicate that heart failure is the primary cause of death in weeverfish intoxication The electrocardiograms taken during the various experiments show pronounced ischaemic changes in the myocardium, ending in ventricular flutters of such severity that life ends The finding on autopsy of severe stasis, particularly in lungs and liver, together with the observation that animals intoxicated by weever venom often die of lung oedema, adds support to this theory The fall in blood pressure occurring after injection of weever venom is probably also due to heart failure The hyaluronidase effect with a reduced circulating blood volume may also be a contributory factor

In addition there could be yet other causes of death, i e the effect on the respiration BRIOT (1902a) gives respiratory paralysis as the cause of death after weever venom injection and supports his theory by the observation that the heart of the animals frequently continues to beat even after respiration has ceased We have seen the same phenomenon and have proved by measurement that both the depth and frequency of respiration were markedly affected after injection of weever toxin, with larger doses there is even transient apnoea as well It has been suggested that accumulation of CO_2 due to tissue ischaemia may be responsible for this but that the extent of haemolysis with loss of O_2 transport may also be a contributory factor However, a more direct neural effect on the respiratory organs cannot be excluded The contractions of the heart that may generally be seen terminally in both human subjects and animals after respiration ceases cannot be taken as an indication of respiratory death, since such contractions can also be observed in death from heart failure These contractions are, however, irregular with highly insufficient cardiac output

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From the Research Division of Pharmacia Ltd, Copenhagen
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Biphasic Response of Guinea-pig's Taenia coli Induced by 1,1-Dimethyl-4-phenylpiperazinium Iodide (DMPP)

By

Jan Weis

(Received March 12 1962)

The guinea pig's Taenia coli is generally supposed to be a ganglionfree smooth muscle However, if the muscle is isolated in such a way that the underlying connective tissue is included, a preparation containing ganglia is obtained The term "taenia coli" refers below to this preparation

With the initial purpose of studying the usefulness of this preparation for screening drugs in vitro, several experiments were carried out in which the taenia coli, with connective tissue, suspended in an aerated Tyrodes bath was subjected to the influence of different drugs active on the autonomic nervous system It was found that the preparation is unsuitable for screening purposes on account of its unstable tonus and the frequent appearance of spontaneous rhythmic activity

The preparation would be expected to contract after application of DMPP (1 1 dimethyl-4 phenylpiperazinium iodide), commonly considered a somewhat specific ganglion stimulant However, it was observed that the taenia coli frequently shows a biphasic response to this agent This paper describes the phenomenon and its modification by other drugs

Methods

Guinea pigs of either sex and weighing 400–500 g. were used The [illegible] underlying connective tissue was disse[illegible] strips of equal length by a transverse [illegible] tion of the composition NaCl 8 g, K[illegible] g NaH_2PO_4 $2H_2O$ 0.06 g [illegible] 0.4 g, Mg[illegible] was aera[illegible] 37 C U[illegible] to front[illegible] up simu[illegible] compounds used were 1,1-dime-

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Ske [illegible]

Skeie [illegible] følgetilstande *Nord Med* 1962b in press

Fig 1 Two different types of response to DMPP
a relaxation with subsequent contraction b contraction with subsequent relaxation
D DMPP 5×10^{-6}. Washing at ●

Fig 2 The effect of hexamethonium (C 6) on
the response to DMPP a The DMPP response before C 6 b The DMPP-response in presence of C 6 5×10^{-6}

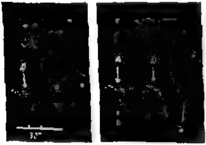


Fig 3 Reversal of the DMPP response by atropine a DMPP alone
b DMPP in presence of atropine 5×10^{-8} Washing at ●

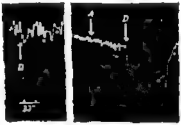


Fig 4 Unmasking by atropine of a relaxation
as response to DMPP a DMPP alone b DMPP in presence of atropine
2×10^{-7} Washing at ●

Fig 5 The effect of yohimbine on the DMPP response
a DMPP before yohimbine b DMPP in presence of yohimbine 1×10^{-5} c DMPP after yohimbine is washed out. Washing at ●

thyl-4 phenylpiperazinium iodide (DMPP) hexamethonium bromide (Vegolysen ® M & B), reserpine (Serpasil ®, Ciba), atropine sulphate, noradrenaline monotartrate tyramine monochloride, yohimbine chloride All doses and concentrations given below refer to the salts

Pretreatment with reserpine was carried out with different doses, and the duration of the treatment was changed from one experiment to another When no other figures are given the dose was 5 mg/kg 17 hrs before the animals were killed

Results

Figure 1 shows the effect of DMPP on the taenia coli preparation in figure 1 a there is relaxation followed by a contraction; in figure 1 b the result of another experiment shows a contraction preceding a relaxation One of the phases may dominate more or less over the other preparations showing a pure relaxation or a pure contraction as well as "a balanced non response" (in which treatment with other drugs would elicit one effect or the other) have also been seen

The pattern of response of one particular strip of gut remains essentially unchanged irrespective of the dose of DMPP, in contrast to the results of GILLESPIE & MACKENNA (1960) with the rabbit colon The only effect of varying the DMPP dose was an increase or decrease in the magnitude of the contraction or the relaxation or both The concentration of DMPP was, therefore, standardized at 5×10^{-6}, and this concentration was used in all experiments referred to later in this paper

The motor as well as the inhibitor component of the response was reduced or abolished by hexamethonium at a concentration of 0 2–10×10^{-6} (fig 2)

The contraction was diminished, abolished or reversed by atropine at concentrations of $5–20 \times 10^{-8}$ (fig 3), the relaxation being unchanged or increased Sometimes with an apparent lack of response to DMPP, a pure relaxant effect was unmasked by atropine 2×10^{-7} (fig 4)

The relaxation was decreased by yohimbine 1×10^{-5} (but never completely abolished (fig 5)) and pretreatment of the animals with reserpine at various doses before removal of the gut also clearly altered the response to DMPP in the direction of a pure contraction However, the relaxation seldom disappeared completely

The Action of Tyramine and Noradrenaline

The results so far obtained had led us to believe that the isolated taenia coli contains an adrenergic mechanism that can be stimulated via nervous pathways This mechanism was then investigated with special reference to the uptake and release of catecholamines Addition of tyramine to the bath (5×10^{-6}) was usually without effect However, occasionally a small

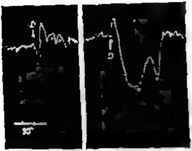
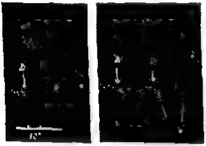


Fig 1 Two different types of response to DMPP
a relaxation with subsequent contraction b contraction with subsequent relaxation
D DMPP 5×10^{-6}. Washing at ●

Fig 2 The effect of hexamethonium (C 6) on the response to DMPP a The DMPP response before C 6 b The DMPP-response in presence of C 6 5×10^{-6}



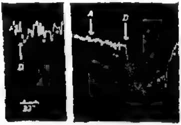


Fig 3 Reversal of the DMPP response by atropine a DMPP alone
b DMPP in presence of atropine 5×10^{-8} Washing at ●

Fig 4 Unmasking by atropine of a relaxation as response to DMPP a DMPP alone b DMPP in presence of atropine 2×10^{-7} Washing at ●

Fig. 5 The effect of yohimbine on the DMPP response
a DMPP before yohimbine b DMPP in presence of yohimbine 1×10^{-5} c DMPP after yohimbine is washed out Washing at ●

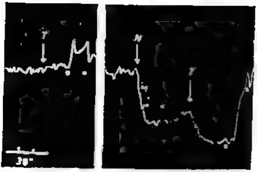


Fig 6 The effect of tyramine (T) 5×10^{-6} a before noradrenaline b in presence of noradrenaline (N) 1×10^{-7} Washing at ●

slow decrease in tonus was observed. It therefore seems unlikely that a release of noradrenaline or of other catecholamines is caused by addition of tyramine. Tyramine and other "neuro-sympathomimetics" are generally supposed to release catecholamines from storage in different organs. This effect disappears after treatment with reserpine, but can be restored by an infusion of noradrenaline (BURN & RAND 1958). Noradrenaline (1×10^{-7}) caused the isolated taenia coli to relax, but after a short time the tonus was slowly re-established. If tyramine was then given a clear secondary relaxation was seen (fig 6), but if the preparation was washed between the addition of noradrenaline and the addition of tyramine, no effect of the latter substance was seen. In a similar manner a previous addition of noradrenaline to the bath to some extent augmented a small inhibitory response to DMPP (as also by this procedure a small relaxation can be elicited besides a pure contraction). Here again washing after adding noradrenaline will eliminate its influence on the DMPP response.

In experiments in which the animal was pretreated with reserpine we also attempted to restore the inhibitory response to DMPP by incubating the preparation with noradrenaline (1×10^{-6} for 20 min). No change was observed when washing preceded the addition of DMPP.

However, in a single experiment with uptake of noradrenaline apparently positive results were obtained. The animal was pretreated with reserpine for 4 days before the experiment (0.5–0.5–2–2 mg/kg). The preparation was incubated for 25 minutes (1×10^{-6} noradrenaline for 15 min, 1.5×10^{-6} for the last 10 min). Just before adding the noradrenaline, 4 ml of heparinized blood from an untreated animal were added. After the 25 minutes' incubation the preparation was washed and rested for 15 minutes. The response to DMPP was now clearly changed, so that two of the pieces of taenia coli with small biphasic responses now produced large relaxations, in one piece with simple but small relaxations these became clearly enlarged and in the last piece, which had previously produced simple contractions, the response to DMPP was reversed into simple relaxation. Treatment with heparinized blood alone did not seem in any way to change the responses. This observation has not been followed further.

After adding tyramine in high doses (concentration $2\text{–}5 \times 10^{-5}$), a contraction of the muscle was often seen When DMPP was given while tyramine was present in the bath the inhibitory phase of the DMPP response was reduced, abolished or even reversed The motor response to tyramine was also present after treatment with atropine and appeared to be more powerful in experiments with reserpine treated animals This effect of tyramine, therefore, must be due to action on the muscle cell itself Contraction of the intestinal smooth muscle in response to tyramine is seen in experiments with the isolated guinea-pig jejunum (SZERB 1961), and FURCHGOTT (1960) suggests that tyramine has some direct action on the smooth muscle cells in isolated arterial strips when given in high doses

Discussion

The contraction of intestinal smooth muscle by DMPP is generally considered to be due to a stimulation of the intramural parasympathetic ganglia of the gut

The relaxing effect of DMPP on the taenia coli preparation, discussed in this paper, could be due to

1 action on the smooth muscle itself,
2 auto inhibition,
3 presence of intramural neurones acting by liberating noradrenaline when stimulated with DMPP in the synapses,
4 postganglionic action of DMPP on adrenergic neurones, nerve endings or chomaffin cells

The first possibility can probably be disregarded, since both the relaxation and contraction of the isolated taenia coli in response to DMPP can be abolished by hexamethonium This suggests a nervous pathway for both phases WINBURY (1959) suggests a direct effect of DMPP on the vascular smooth muscle of the dog's hind limb, but this relaxation is hexamethonium resistant

Auto inhibition is described by BIJLSMA (1961) in some experiments with valerylcholine This substance is supposed to stimulate the parasympathetic ganglia in the same way as acetylcholine and also to exert a competitive antagonism against acetylcholine liberated from the nerve endings As the inhibitory action of DMPP on taenia coli decreases or disappears after hexamethonium, this action is likely to have a nervous pathway In the presence of large doses of atropine supposed to exert complete peripheral blockade of the action of acetylcholine liberated by stimulation of the ganglia, it is still possible to elicit a relaxation with DMPP The use of anti adrenergic substances causes the inhibitory phase of the response to DMPP to decrease These three experimental facts seem

Fig 6 The effect of tyramine (T) 5×10^{6} a before noradrenaline b in presence of noradrenaline (N) 1×10^{7} Washing at ●

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to ATP. However, STJARNE (1961) has shown that the catecholamine content of venous blood from the cat's adrenals was unaltered or even reduced after administering tyramine. In the venous blood from the spleen the catecholamine concentration was increased and he argued that granules from different organs are different in accessibility or sensitivity to tyramine. The result of the one above mentioned experiment with blood in the bath fluid might be explained by assuming that the blood contains a factor (possibly an enzyme system) necessary for the transport of noradrenaline though the cell membrane to the storing granules or that it contains a "storage factor". One or both of these factors could be present in some organs, e. g. in the atria of the rabbit (BURN & BURN 1961), and not in others. MUSCHOLL (1960), however, failed to show any uptake of noradrenaline by the heart of reserpinized rats after noradrenaline infusion, he therefore concluded that the reappearance of tyramine action found by BURN & RAND (1958) after infusion of noradrenaline to reserpinized animals must be explained by assuming that tyramine can act only when noradrenaline is present, a theory confirmed by the findings on the taenia coli. In reserpine pretreated animals reserpine will be present in the system, and this may prevent the uptake of noradrenaline, i. e. intracellular accumulation by binding to ATP. In experiments with isolated organs the reserpine will disappear sooner or later, and the possibilities for uptake of noradrenaline thus return.

Summary

Experimental evidence for a special autonomic mechanism in the isolated taenia coli of the guinea pig is presented. This mechanism makes possible that one stimulating substance should at the same time elicit an adrenergic relaxation and a cholinergic contraction.

The adrenergic mechanism is not directly accessible to tyramine (no liberation of noradrenaline), and no uptake of noradrenaline to the original sites can be shown under normal experimental conditions. It seems that the conditions can be altered so that an uptake is possible (blood in the bath fluid). Under normal experimental conditions, however, some noradrenaline can be loosely bound by the preparation and liberated by tyramine.

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to show that the complex effect of DMPP on the preparation mentioned here cannot be due to auto inhibition

It is considered that the relaxing effect of DMPP is due to interference with adrenergic structures as suggested in 3 and 4 above It is not possible to decide between these two possibilities on the basis of the results obtained during the work discussed here However, it should be pointed out that an explanation for a mechanism of type 3 has been given by AMBACHE (1951) for the action of nicotine on the gut segment from mice and rabbits pretreated with botulinum toxin and by AMBACHE & EDWARDS (1951) for the action of nicotine on the atropinized isolated gut and stomach strip of the kitten This relaxation was abolished by hexamethonium and by large doses of nicotine, and it was therefore concluded that both the motor and inhibitor response were elicited via the ganglia

The mode of action suggested under 4 above is more in line with the results of GILLESPIE & MACKENNA (1960, 1961), who have shown an overlapping of the parasympathetic and sympathetic system in the isolated rabbit colon

The observed effect of tyramine in the presence of noradrenaline could indicate a binding of the noradrenaline to receptors other than those through which the effect on the muscle cell is elicited This binding does not seem to be strong being abolished by washing the preparation As the relaxing part of the DMPP response in some experiments seems to increase when noradrenaline is present in the bath, it might be supposed that these storage receptors, or at least some of them, have an intimate connection with the nerve endings This is in agreement with the theories of STJARNE (1961), who suggests that the storing receptors are probably situated on the surface of the nerve fibre itself MUSCHOLL (1961) postulates that only noradrenaline bound to the effector receptors is metabolized, the stored noradrenaline being protected against metabolism Another possible explanation is that the noradrenaline present spatially occupies the structures involved in the elimination mechanism (storing receptors enzyme systems, etc), so that a given dose of liberated noradrenaline can act for a longer time at higher concentration resulting in increased relaxation on addition of DMPP On the basis of their conclusion from experiments with isolated atria of the rabbit incubated with ^{14}C noradrenaline, BURN & BURN (1961) suggest that a part of this is taken up in intracellular stores from which it can be directly liberated by tyramine As tyramine alone in small doses had no effect on the isolated taenia coli (or only an insignificant effect) it can hardly be able to invade the nerve endings and liberate catecholamines from granules there A liberation of catechola

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Toxicological and Pharmacological Criteria of Repeated Doses of a Hepatotoxic Agent

By

C. S Weil and M E Goldberg

(Received March 12 1962)

The role of pharmacological intervention as a tool for studying potential agents of liver injury has gained wide acclaim in recent years (PLAA *et al* 1958 CALVERT & BRODY 1960 and FUJIMOTO & PLAA 1961) Many of these studies have used animals acutely treated with a hepatotoxic agent that manifested only early signs of histopathological derangement This investigation has been undertaken to assess the importance of such intervention in a subacute feeding study of such an agent

BENNETT, DRINKER & WARREN (1938) noted that chlorinated naphthalene and diphenyl compounds could produce marked liver damage in the rat without any demonstrable microscopic changes in other organs All portions of the liver lobule were involved, despite the fact that initial morphological changes were more pronounced in the parenchymatous cells nearest the hepatic veins The successive degenerative changes were cloudy swelling, fatty infiltration, fatty degeneration and, finally, complete disintegration of the cell They noted that widespread degenerative changes of all liver cells could exist for several months without producing any evidence of ill health in the animals

Unpublished results from our laboratory (SMYTH & WEIL 1947) have shown that hexachloronaphthalene, 61 % chlorine, hereafter referred to as HEXE is a potent hepatotoxic agent of the type discussed above The inclusion of 0 02% of this chemical in the diet for 90 days produced microscopic evidence of fatty degeneration in the liver, associated with mean liver weight (as percentages of body weight) in male and female rats of 171 and 187%, respectively, of the means of their controls A concentration of 0 002% in the diet of this material did not produce significant liver weight or micropathological alteration

These two concentrations, 0 02 and 0 002%, and an intermediate one 0 0063%, were selected for inclusion in a 12 week feeding study to compare the usefulness of pharmacological measurements as a means of

evaluating liver toxicity The response of treated animals to a depressant of the central nervous system and to a stimulant have been compared by use of several usual toxicological parameters of injury diet consumption body weight gain, liver weight and kidney weight It was considered redundant to repeat the micropathological examination of the livers of the rats, as this has been so amply described by BENNETT *et al* (1938) It has, further, been reported (SMYTH *et al* 1952, ROWE *et al* 1959) that the weight of the liver is often significantly increased at dosage levels below those causing micropathological changes

Prolongation of the barbiturate anaesthesia by a pharmacological active agent may be caused by several factors Usually it is the result of *potentiation of the barbiturate depressant effects by a central nervous* action or to an inhibition of the metabolism of the barbiturate by a direct interference in the liver with liver enzyme systems To determine the central depressant effect of HEXE, the responsiveness to pentetrazol = pentamethylenetetrazole was investigated LU *et al* (1961) have shown that rats receiving small concentrations of sodium fluoride in the diet are more sensitive to the effects on the central nervous system of stimulants and depressants Our investigations has included NaF, therefore as a positive control

Methods

The rats used in this study were females from a colony originally stocked and currently maintained with the Carworth Farms Elias (CF–E) strain Five dosage groups and one sub-group (B) were used

Code in Table 1	Material	% in Diet
A	Hexachloronaphthalene (HEXE)	0 02
B	HEXE	0 02 for first 4 weeks control diet next 4
C	HEXE	0 0063
D	HEXE	0 002
E	Sodium fluoride (NaF)	0 015
F	Control diet (ground PURINA rat chow)	

Group A was randomly divided at the end of four weeks dosing half remained on 0 02% HEXE for the next four weeks and the other half was placed on control diet for the same period (Group B) All of the other groups received their diets for a maximum of 12 weeks dosing

Each group consisted of 104 rats 40 of each were approximately 39 days of age when dosing began (termed older rats hereafter) and 64 were approximately 32 days of age at this time (younger rats) This was made necessary because of the large number of rats required The results for each criterion of effect were compared separately for each age group and combined except for body weight gain as the variances were otherwise homogeneous The rats were assigned to their groups on the first day of

dosing completely at random They were weighed after four days on their diets and at weekly intervals thereafter On the day before the end of 1, 4 and 8 weeks of dosing 6 rats from each group were randomly selected for use as described below

(a) Either 6 or 8 per group received one intraperitoneal injection of 72 mg/kg of hexobarbital[1] (24 mg/ml in 0 9% saline) Sleeping time for each rat was defined as the time between loss of righting reflex and its return The end point was the ability of the animals to right themselves when placed on their backs on a flat surface Rats that did not sleep were excluded from statistical comparisons

(b) Three sub-groups 4 to 7 rats each per dosage level received one intraperitoneal injection of pentylenetetrazol (13 33 mg/ml of 0 9% saline) forty mg/kg in saline was given intraperitoneally to one sub-group of each dosage level group An all-or-none response for a reaction was obtained for each rat To prevent stimulation from aggregation effects (GUNN & GURD, 1940), each rat in the pentylenetetrazol study was housed individually after injection A positive end point was the presence of at least one well-defined convulsion in the animal Tremors were not considered as a positive response Depending upon the number of animals responding to a 40 mg/kg dose, the next two groups were given the stimulant in doses greater or less than the original one by a logarithmic factor This procedure was done to ensure a graded response that should encompass the 50% response level The median response (CD_{50}) was calculated by the method of moving averages (WEIL, 1952) when the number of rats per sub-group was constant Otherwise the method of Lichtfield and Wilcoxon (1949) was used

The morning after the injections these randomly selected rats were weighed They were then killed and bled by sectioning the cord and neck vessels without cutting the trachea and suspended by their tails until heart action had ceased The liver and kidneys of each rat were removed and weighed to the nearest 0 01 gram These organ weights were converted to percentages of body weight for each rat

Bartlett s method (1937) was used to compare variances for each criterion of effect, with the exception of the pentetrazol stimulation described above If variances were heterogeneous individual F ratios were calculated for each intergroup comparison and the proper t test was applied If they were homogeneous, analysis of variance was used When this indicated significant intergroup differences these were del [illegible]

[illegible] week periods

Results

[illegible]

it

Ne

Sn [illegible] effect or definite trend of differences in excitation was seen after intraperitoneal injection of pentetrazol The CD [illegible]

[1] Kindly supplied by Dr G H WESSINGER Sterling Winthrop Research Institute, Rensselear New York

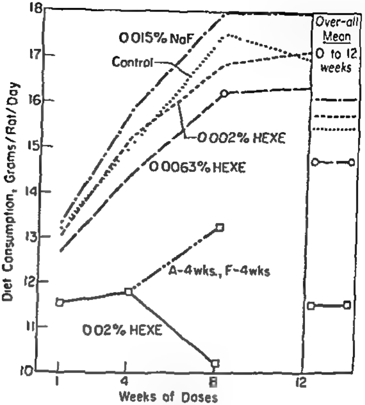

Figure 1 Mean Diet Consumption of Rats
Legend
versus control
○ 0 05 > p > 0 01 A 0 02% HEXE
□ p < 0 001 F control

The gain in body weight of the younger and older rats is shown in figure 2 In this and other graphs the mean response of each group is indicated, as is the statistical significance of the difference from the controls. Intergroup differences in body weight and the other measurements are presented in table 1.

The NaF-fed rats gained at least as much weight as did their controls over all the periods measured At eight weeks, the older NaF rats gained significantly more weight than the controls At no other periods were the differences significant for either the younger or the older rats

The HEXE-fed rats, however, showed depression of body weight from the first measurement (4th day) onwards The greater weight depression *was seen in the younger rats The weight depression was proportional* to the dosage, *e g*, from the first week through 8 weeks of dosing (in this younger group) Group D differed significantly from group C and group C significantly from group A As group A, 0 02% HEXE, produced an enormous effect on this and other values almost immediately, it was subdivided at the end of 4 weeks, and half of the surviving rats were placed on control diets. *Four weeks later this group (B) had gained*

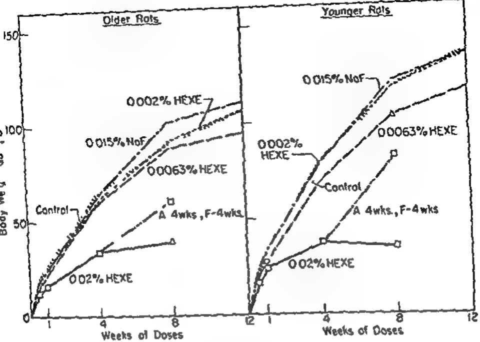

Figure 2 Body Weight Response of Rats
Legend
versus control
0 01 > p > 0 001 A 0 02% HEXE
p < 0 001 F control

significantly more weight than its counterpart (A), but had not achieved body weight similar to that of the control rats (F) or those in the NaF or other HEXE groups (C, D or E)

Similarly liver weight expressed as a percentage of body weight, was a sensitive criterion of effect Here again, the NaF rats did not differ from the controls All HEXE groups differed from the control from the one week examination period to the end of the study Further, as with body weight a graded response was seen, in that all the HEXE groups differed from one another The higher the dosage level the higher the increase in this liver weight body weight ratio

Kidney weight on the other hand, was not especially sensitive None of the groups differed among themselves after one week of dosing, for all practical purposes none but the 0 02% HEXE group (A) differed at any time in the study As for body weight the B group (0 02% for 4 weeks, control for 4 weeks) showed recovery over A, but still differed significantly from A and from all of the other groups in liver and kidney weights

At the end of the first week of doses, only the NaF group had after hexobarbital a prolonged sleeping time significantly different from the

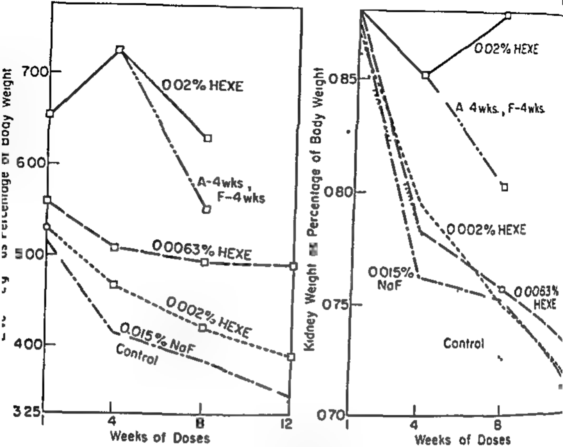

Figure 3 Response of Liver Weight and Kidney Weight
(as Percentage of Body Weight)
Legend
versus control
○ 0 05 > p > 0 01 A 0 02% HEXE
□ p < 0 001 F control

control group, F, and it remained different from this group throughout the study Though group A was not different at one week, it exceeded all *other groups at 4 and 8 weeks At this time group B showed almost* complete recovery It had a significantly lower sleeping time than groups A and E (0 02% HEXE and 0 015% NaF) and one not different from that of the control (F) Further, the middle concentration of HEXE (0 0063% – group C) did not differ in effect from the controls until the 12th week At this time its effect was also significantly higher than that of the lowest HEXE dosage, in group D

Discussion

No change in the sensitivity to pentetrazol induced convulsions was observed in any of the treatment groups during the 12-week study From these results it may be inferred that HEXE possessed no marked

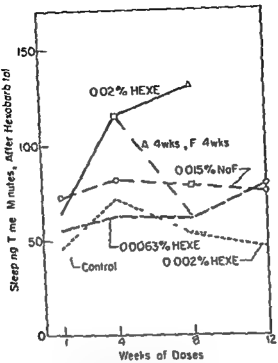

Figure 4 Responses of Rats to Hexobarbital

Legend
versus control

○ $0\ 05 > p > 0\ 01$ A 0 02% HEXE
△ $0\ 01 > p > 0\ 01$ F control
□ $p < 0\ 01$

central depressant effects Thus it is probable that the increase in sleeping time caused by HEXE after a barbiturate is a manifestation of hepatotoxic mechanisms The 0 02% level apparently raised the sleeping time after one week but this elevation was not statistically significant from that in the control group The criterion might have been more sensitive if more animals had been tested per dosage group After an additional three weeks on the diet the 0 02% group was found to differ highly significantly from the control group Only after 12 weeks of diet did the 0 0063% HEXE group show any difference from the controls in sleeping time The sodium fluoride group showed a potentiation of barbiturate sleeping time from the first week on without any evidence of hepatotoxic effect These results confirm the report of Lu *et al* (1961) However we could not confirm their results showing an enhancement of pentetrazol response after feeding 150 ppm of added NaF They also reported this effect after daily intraperitoneal injections of 10 mg/kg/day NaF for 11 weeks

After the first four weeks of study half of the 0 02% HEXE group was

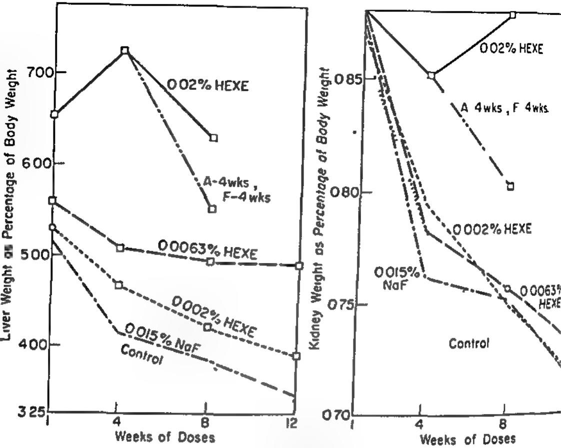

Figure 3 Response of Liver Weight and Kidney Weight (as Percentage of Body Weight)

Legend

versus control

○ 0 05 > p > 0 01 A 0 02% HEXE

□ p < 0 001 F control

control group, F, and it remained different from this group throughout the study Though group A was not different at one week it exceeded all other groups at 4 and 8 weeks At this time group B showed almost complete recovery It had a significantly lower sleeping time than groups A and E (0 02% HEXE and 0 015% NaF) and one not different from that of the control (F) Further, the middle concentration of HEXE (0 0063% – group C) did not differ in effect from the controls until the 12th week At this time its effect was also significantly higher than that of the lowest HEXE dosage, in group D

Discussion

No change in the sensitivity to pentetrazol induced convulsions was observed in any of the treatment groups during the 12 week study From these results it may be inferred that HEXE possessed no marked

given the control diet After an additional four weeks on control diet these animals showed a return to normal in their barbiturate response They did not recover, however, in body weight gain, liver weight or kidney weight BENNETT *et al* (1938) further noted that liver cell changes were still present after a two-months recovery period and quoted this as evidence that this type of injury is persistent, with only slow recovery from it

It is apparent, from examination of Table 1 and Figures 1 to 4, that gain in body weight, and liver weight as a percentage of body weight are sensitive criteria for this type of liver involvement Kidney weight and sleeping time were affected later and to a less extent Diet consumption was even less sensitive than kidney weight and sleeping time

Summary

In a subacute feeding study with a hepatotoxic agent (hexachloronaphthalene), pharmacological measurements showed significance only after several of the toxicological signs of injury had been observed Food consumption liver weight and body weight were all significantly different from those of the control group within one week of diet Prolongation of barbiturate sleeping time" was first observed at four weeks, as was increased kidney weight

The only criterion of effect altered in rats eating diets containing sodium fluoride was barbiturate potentiation This first occurred in one week Depression of the central nervous system, as measured by pentetrazol stimulation was not altered by either material

Although body, kidney and liver weights were only partially restored to normal when the rats previously on the highest level of hexachloronaphthalene were placed on the control diet for four weeks, the response in this group to the barbiturate was normal Thus, a comparison of such tests in a feeding study with an agent of liver injury indicates that pharma cological intervention with a barbiturate shows significant effects only after clear evidence of damage exists and returns to normal before the damage is repaired

Acknowledgements

The authors express their appreciation to M WOODSIDE and J BERNARD for assistance in the preparation of the diets involved and the care of the animals N CONDRA and J STRIEGEL assisted in killing and autopsy of the rats and weighing of their livers and kidneys H E JOHNSON provided valuable technical assistance in the pharmacological phase of this investigation

Table 1.

Inter-group Comparisons

Number of days or weeks in the diet	HEXE % in diet and code[1] 0 02 A	0 02[2] B	0 0063 C	0 002 D	NaF 0 015 E	Number of rats per group
	Body weight gain, older rats					
4 days	(EF)c	–	EaFc	Fc		39 to 40
1 week	(CDEF)c	–				39 to 40
4 weeks	(CDEF)c	–				27 to 33
8 weeks	(CDEF)c	(CDEF)c	Ea	Ea	Fa	8 to 20
12 weeks	–	–				8 to 11
	Body weight gain, younger rats					
4 days	(DE)cFb	–	(DE)b			62 to 64
1 week	Cb(DEF)c	–	DaEbFa			61 to 64
4 weeks	(CDEF)c	–	(DE)a			43 to 49
8 weeks	(BCDEF)c	Cb(DEF)c	Da(EF)b	Ec		10 to 35
12 weeks	–	–	Fa			12 to 16
	Liver weight as percentage of body weight					
1 week	(CDEF)c	–	DaEbFc	Fa		25 to 26
4 weeks	(CDEF)c	–	(DEF)c	(EF)c		24 to 26
8 weeks	Bb(CDEF)c	Cb(DEF)c	(DEF)c	(EF)c		15 to 25
12 weeks	–	–	(DEF)c	(EF)c		21 to 25
	Kidney weight as percentage of body weight					
1 week		–				25 to 26
4 weeks	Cb(DEF)c	–				24 to 26
8 weeks	Ba(CDEF)c	(CDE)bFc	Fa			15 to 25
12 weeks	–	–				21 to 25
	Sleeping time					
1 week		–			Fa	3 to 6
4 weeks	(CDE)bFc	–			Fa	4 to 7
8 weeks	(BCDEF)b	Ea	Eb	Ec	Fc	5 to 7
12 weeks	–	–	(DF)a	Eb	Fc	3 to 6

1 Capital letters are code designations for various dosage groups Code for control group = F

2 0 02% for first 4 weeks control for next 4 Lower case letters indicate probability of differences between groups

a = 0 05 > p > 0 01
b = 0 01 > p > 0 001
c = p < 0 001

... the same stat st cal significance
t to left, e g , to compare
does not appear under E,

– group not in existence at time of comparison

given the control diet After an additional four weeks on control diet these animals showed a return to normal in their barbiturate response They did not recover, however, in body weight gain, liver weight or kidney weight BENNETT *et al* (1938) further noted that liver cell changes were still present after a two months recovery period and quoted this as evidence that this type of injury is persistent, with only slow recovery from it

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Acta pharmacol et toxicol 1962, 19, 139–145.

From the Laboratory of Plant Physiology (Professor E K Gabrielsen, D Sc) and the Department of Pharmacology and Toxicology (Professor Sv Dalgaard Mikkelsen, V M D), Royal Veterinary and Agricultural College, Copenhagen

Plant Hormonal Effect of Serum and Urine Before and After Adding Indole-3-Acetic Acid

By

A. Skytt Andersen and Folke Ronnike

(Received March 12 1962)

Indole-3-acetic acid (IAA) is an integral part of human serum and urine and is responsible for their auxin effect (KOGL, HAAGEN-SMIT & ERXLEBEN 1934, BENNET CLARK *et al* 1952, RONNIKE 1960) IAA in serum is to a large extent bound to proteins (RONNIKE 1959) To investigate whether serum *in vitro* exhibits any binding capacity for added IAA, the auxin effect of IAA at various concentrations was examined after adding 1% human serum Further experiments were designed to show the change in auxin effect of serum and urine of rats after *in vivo* treatment with high concentrations of IAA

Materials and Methods

The auxin effect was assayed biologically by the method of BURSTROM (1960) wheat seeds (variety "Svalof 170", harvest 1959) surface sterilized for a two hour imbibition period with a solution of 200 ppm formaldehyde mixed with 100 ppm TWEEN 20 (polyoxyethylene sorbitan monolaureate), were placed in large petri dishes on several layers of wet filter paper and germinated in the dark at 23°C for 48 hours Then seedlings of uniform root length (15–20 *mm*) were selected and transferred to plastic holders in 20 or 30 ml pyrex beakers, each containing 5 ml solution Transfer [illegible]

was used either a [illegible]

KH_2PO_4 2×10^{-4} [illegible]

water to which the various materials (human or rat serum, rat urine, indole 3 acetic acid) were added Twelve plants were employed in each test (4 replicate beakers containing 3 plants each), all of the tests were repeated at least 3 times in independent experiments The plants were grown in the test solution for 24 hours under the same conditions as during germination [illegible]

each plant was excised, and the l[illegible]

from the apex was measured mic[illegible]

sured, and the analysis of variance, which was calculated for each experiment generally showed 95% confidence limits to be $\pm$ 5–8 μ

Indole-3-acetic acid ("Hoffmann la Roche" or "Merck Chem") was made up in $10^{-4}M$ solution in a weak solution of NaOH The solutions required for experiments were made by dilution of this stock solution with redistilled water and adjusted to pH 5 with HCl The calculation of IAA equivalents was made by estimations of ED 50 *i e* effective dosage for 50% inhibition, (HORSFALL 1953) This concentration could then be compared with that of solutions containing unknown concentrations of IAA Thus,

$$\frac{\text{ED 50 IAA}}{\text{ED 50 UK}} \times 100$$

when ED 50 IAA is in mol/liter and ED 50 UK (UnKnown) is as a percentage the resulting approximation to the IAA equivalent is in mol/liter

Human blood samples were drawn from normal healthy persons within 24 hours of beginning each experiment and stored at 4°C The blood was centrifuged at 4000 rotations per minute for 20 minutes, and the serum was diluted to the desired concentration with nutrient solution or redistilled water

In the rat experiments young male rats weighing 90–150 g each were used Before the experiment the animals were kept without food for 24 hours Generally all the blood (4–5 ml) was drawn from one animal for one experiment The rats were anaesthetized by CO_2 inhalation, and the blood was taken from the aorta Fractionation of blood cells and serum was done by centrifugation, as with human blood The IAA treatment of the rats was carried out by the method of MIRSKY *et al* (1956) The dosage was 0 4 mM IAA per 100 g body weight The solution used for treatment contained 0 4 mM/5 ml and was prepared immediately before administration by dissolving the IAA in 0 5% $NaHCO_3$ solution with a few drops conc NaOH solution added The solution was adjusted to pH 8, and 5 ml/100 g body weight were administered to the rats by stomach tube No directly observable effect of this treatment was evident For the serum tests the blood was then taken from the rats after 1 or 1½ hours and stored at 4°C until the plant test began Generally this storage period did not exceed 12 hours In the urine experiments the rats were treated similarly with IAA and then enclosed in small cages from which the urine was collected though a funnel in test tubes kept cold by ice in thermos flasks

Identical procedures omitting the IAA treatment, were employed with other rats to secure comparative values for untreated rat serum and urine

Results

Root-cell elongation experiments The experiments were carried out over a period of 7 months, in order to evaluate the inter-experimental variation, cell-lengths of roots grown in redistilled water or in nutrient solution were recorded in each experiment For water the 70 values varied between 227 μ and 315 μ with a mean of 271 $\pm$ 6 μ, for nutrient solution the cell-lengths in 56 observations were between 196 and 268 μ and the mean was 239 $\pm$ 5 μ The results were recorded only as cell lengths in μ

Root-growth inhibition effects of human serum were re-established with our technique at concentrations ranging from 0 5 to 5% The serum was

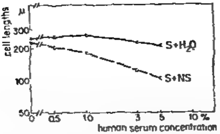

Fig 1 Inhibition of epidermal root cell growth by different concentrations of human serum diluted with redistilled water (S + H_2O) or with nutrient salt solution (S + NS) The points represent means ±95% confidence intervals in various experiments (inter-experimental variation)

added to redistilled water as well as to nutrient solution, and cell growth was inhibited as shown in fig 1 When serum was diluted with distilled water, ED 50 (50% inhibition) was not reached, but extrapolation of the curve indicated the IAA equivalent of native human serum to be ca 4×10^{-7}M If, however, the serum was diluted with nutrient salt solution, the inhibition was greater, the ED 50 was 4%, resulting in an IAA equivalent for this human serum diluted with salt solution to be 6×10^{-6}M IAA

To establish a concentration response curve for root cell elongation to exogenous applied IAA, experiments were carried out with different concentrations of IAA added to the nutrient solution The results, illustrated in fig 2, show a linear relationship between log concentration and log cell-length within the concentration range covered by this graph

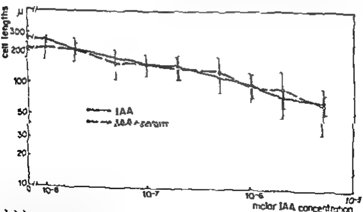

Fig 2 Inhibition of epidermal root cell growth by indole 3 ...
3 acetic acid +1%, human ser...
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each concentration

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Identical procedures omitting the IAA treatment were employed with other rats to secure comparative values for untreated rat serum and urine

Results

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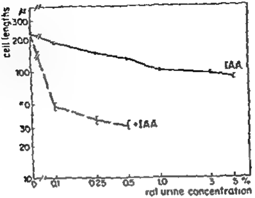

Fig 4 Inhibition of root-cell growth by different concentrations of rat urine diluted with

the two lower curves in fig 3 The IAA equivalent calculated from these curves for rat serum 60 minutes after administering the solutions was 8×10^{-5}M, after 90 minutes it had risen to 2.5×10^{-3}M

Experiments with rat urine Since a certain amount of auxin is normally known to be excreted in human urine, the auxin effect of rat urine from normal untreated and from IAA-treated rats was assayed The IAA treatment was similar to that described for the serum tests Urine from 15 rats was collected over 24 hour periods, after treatments, in individual containers kept at 0–4°C Each portion of urine was diluted with redistilled water to 20 ml, and from this a concentration series ranging from 0.1 to 5% was made by dilution with nutrient solution The responses of wheat root cells to these dilutions are shown in fig 4 Urine from IAA-fed rats had a pronounced inhibitory effect when compared with urine collected from rats under similar conditions, which received no IAA Thus, the estimated IAA equivalent of urine from untreated rats was 2.5×10^{-5}M, whereas it was 8×10^{-3}M for IAA treated rats

Discussion.

Investigations similar to those reported in this paper have, as far as we know not been previously performed Whether the auxin present in the sera, as indicated by this and previous experimental evidence, has any implications for animal physiology is at present not known The experiments showed that the auxin effect of the native plant hormone indole 3 acetic acid, measured by the inhibition of root cell growth, was not changed by the addition of 1% human serum Human serum diluted

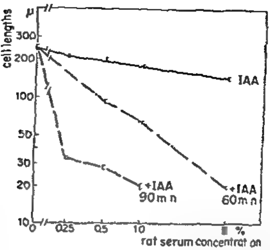

Fig 3 Inhibition of root cell growth by different concentrations of rat serum diluted with nutrient salt solution Serum from rats receiving previously no indole 3 acetic acid (−IAA) a... utes (...sent

There was no inhibition of cell growth at 10^{-8}M IAA, and ED 50 occurred at 2.5×10^{-7}M IAA

In vitro combination of indole 3 acetic acid and human serum The effect of *in vitro* combination of IAA and serum was investigated in a series of experiments in which 1% human serum was added to different concentrations of IAA in nutrient solutions The range of IAA concentrations was from 10^{-11} to 5×10^{-6}M, but only concentrations above 10^{-8}M showed any inhibition of cell growth (fig 2) The results from these experiments exhibit a greater variation than those from the experiments on IAA concentration response presumably owing to the variation in sera but at no point did the IAA + serum curve differ significantly from the IAA concentration curve

In vivo experiments on rat blood Before the *in vivo* experiments on rats the concentration response curve of root cells to native rat serum diluted with nutrient solution was established in the same way as for human serum (fig 3) The salt effect of the nutrient solution was not as pronounced as it was for human serum and generally, rat serum exhibited less auxin effect than human serum the IAA equivalent for rat serum diluted with salt nutrient solution was 1.6×10^{-6}M

To investigate the effect of feeding IAA solution *in vivo*, 6 rats were treated with 0.4 mM IAA/100 g body weight After one hour all blood was extracted from 3 rats At this time only part of the solution had passed from the stomach into the intestine, but when after 90 minutes the 3 remaining rats were killed all the solutions had passed into the intestine The results of auxin assays of sera from both groups of rats are shown in

auxin") aus Harn XI Mitteilung uber pflanzliche Wachstumsstoffe *Zeitschr physiol Chem* 1934, 288, 90–95
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with salt solution exhibits an auxin effect corresponding to an IAA concentration of ca 10^{-7}M, but the auxin effect of serum when water has been used as diluent is somewhat lower, 5×10^{-8}M (fig 1 and RONNIKE 1960) The auxin effect of IAA was not influenced by serum addition in the *in vitro* experiments This possibly indicates that the IAA binding capacity of serum is at a maximum if some form of an equilibrium state exists between free and protein-bound auxin in serum

In vivo treatment of rats with 0 4 mM IAA (= 70 4 mg)/100 g body weight resulted in a pronounced increase in auxin activity of the rat serum and urine The high IAA dosage was used in order to follow the experimental method developed by MIRSKY *et al* (1956) in their investigations on the "hypoglycaemic" effect of various plant growth regulators in experimental animals (Such a "hypoglycaemic" effect of IAA has not been confirmed in a series of experiment performed by one of us – F R in press Binding of IAA in the liver or other organs of rats has not been demonstrated in the present investigations, but the results are not conclusive on this point

Summary

The auxin effect of indole 3 acetic acid at various concentrations as assayed by the wheat root cell elongation test, has been investigated after *in vitro* addition of 1% human serum The auxin effect of IAA did not change after the treatment possibly indicating that the IAA-binding capacity of serum is at a maximum *In vivo* treatment of rats with IAA (0 4 mM/100 g body weight, administered by stomach tube) resulted in a pronounced increase in the auxin effect of blood as well as urine from the experimental animals The experiments indicated that the auxin effect of IAA was not changed by *in vivo* passage through the stomach or intestinal mucus membranes or the liver circulatory system in rats

Acknowledgement

This study has been supported by a grant from The Danish State Research Council

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■ Über ein neues Auxin (Hetero

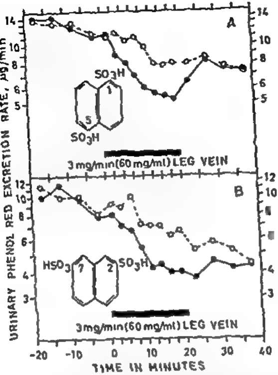

Fig. 1 Effects of unilateral leg vein infusion of 1 5 naphthalenedisulphonic acid (A) and 2,7 naphthalenedisulphonic acid (B) on urinary phenol red excretion Experiment B was done on the same hen 2 5 hours after infusion A Phenol red was given in single doses into the pectoral muscle 1 5 mg/kg one hour before test A and 0 8 mg/kg 50 minutes before test B ● ——— ● phenol red infused side ○ --- – ○ phenol red, control side

NDSA was about the same in one experiment (fig 1A) Gradually smaller differences in phenol red excretion between two sides were observed when 2 7-NDSA was infused in the same way at 2 2, 1 0 and 0 3 mg/min , each dose being used once in a different hen

On the other hand 2,7 NDSA infused unilaterally into the leg vein at 1 0 mg/min for 15 minutes was excreted at a much faster rate on the side of infusion than on the opposite side (fig 2A) After probenecid, the onset of urinary excretion of 2,7-NDSA was delayed, the overall rate of output became smaller, the ipsilateral excess was reduced, and the decay of excretion was more gradual (fig 2B) These findings are consistent with 2 other

probene

extinctic

spondin

The results show that, in spite of strong ionization, 2,7-NDSA

Acta pharmacol. et toxicol. 1962, **19**, 146–148.

From the Department of Pharmacology University of Uppsala, Sweden
(Professor Ernst Bárány, M D)

Secretion of 2,7-Naphthalenedisulphonic Acid in the Chicken's Renal Tubules

By

Bohdan R. Nechay[1]

(Received March 1, 1962)

Introduction

HOBER (1940) found that naphthylamine- and naphtholdisulphonates were not secreted by the renal tubules of the frog In search for a non-toxic substance, convenient to determine at low concentrations and excreted solely by glomerular filtration, the renal excretion of some unsubstituted napthalenedisulphonic acids has been examined in the chicken

Methods

Hens Rhode Island Reds, 2 4–3 2 kg, with the ability to excrete a single unilateral leg-vein dose of 0 3 mg phenol red predominantly on the injected side *Urine collection* separately from two ureters (CAMPBELL 1960) *Infusions* in saline solutions, administered through plastic catheters by motor driven syringes *Phenol red* samples diluted with equal volumes of 1% sodium carbonate, mixed, and their optical density determined in Zeiss Elko II colorimeter with Zeiss Opton filter S 53 51 *1,5-Naphthalenedisulphonic acid (1,5-NDSA) and 2,7-naphthalenedisulphonic acid (2 7 NDSA)* samples diluted with water to obtain final concentrations of 1–3 and 0 5–1 5 μg/ml, respectively then directly read in Beckman spectrophotometer at maxima of extinction, 226 5 (slit 0 2 mm) and 232 0 mμ (slit 0 6 mm), respectively The sodium salts of these acids were obtained from Eastman Kodak and recrystallized several times

Results

Unilateral leg vein infusion of 3 0 mg/min 2,7-NDSA for 20 minutes resulted in ipsilateral depression of urinary phenol red excretion by about 50% in 3 birds (fig 1B) The response to an analogous infusion of 1 5-

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of Pharmacology and ...
ville, Florida

Acta pharmacol et toxicol 1962, 19, 149–155

From the Department of Pharmacology, University of Copenhagen
(Professor Knud O Møller, M D)

The N-demethylation of Morphine in Rats. Quantitative Determination of Normorphine and Morphine in the Urine and Faeces of Rats given Subcutaneous Morphine

By

Kirsten Milthers

(Received April 28 1962)

N de alkylation seems to be a common metabolic pathway for analgaesics It has been demonstrated for several morphine like compounds, for instance codeine (ADLER 1952) and meperidine (PLOTNIKOFF, ELLIOTT & WAY 1952), but evidence for the N demethylation of morphine has been only sparse AXELROD (1956) demonstrated *normorphine* as a morphine metabolite *in vitro*, and MARCH & ELLIOTT (1954) and ELLIOTT *et al* (1954) observed, that $^{14}CO_2$ appears in the expired air shortly after injection of morphine N $^{14}CH_3$ Obviously these observations cannot be considered conclusive evidence However, MISRA, MULÉ & WOODS (1961 b) have recently demonstrated *nor*morphine qualitatively in the urine of rats given morphine

Quantitative recovery of *nor*morphine produced from morphine *in vivo* has not been previously accomplished Using a method recently described (MILTHERS 1961) we have investigated the elimination of systemic morphine as the parent substance and its metabolite *nor*morphine, in the urine and faeces of rats

Methods

Male albino rats were maintained under constant [illegible]

[illegible] period the rats were placed in cages permitting urine and faeces to be collected separately [illegible]

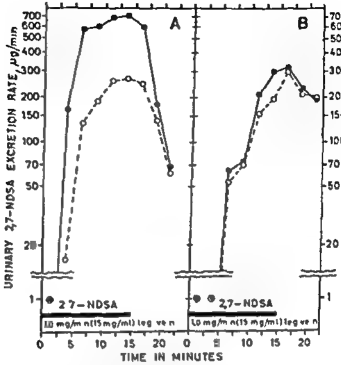

Fig 2 Urinary excretion of 2,7 naphthalenedisulphonic acid (2,7-NDSA) Unilateral leg vein infusion first in normal hen (A) and 2 5 hours later in the same bird treated with probenecid (B) 50 mg/kg into the wing vein one hour before the second infusion ●———● 2,7 NDSA, infused side, O - - - - - O 2,7 NDSA control side

eliminated by the "weak acid mechanism" of the renal tubules in the chicken Since 1,5-NDSA depresses phenol red secretion, it probably also shares the same transport system

Summary

1,5 and 2,7-naphthalenedisulphonic acids diminished tubular secretion of phenol red The secretion of 2,7-naphthalenedisulphonic acid was reduced by probenecid

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urine of rats after subcutaneous morphine

normorphine in the urine								Excreted normorphine as per cent of excreted normorphine + morphine
injection				Total				
4 day		5 day		morphine		normorphine		
M µg	N µg	M µg	N µg	mg	% of inj dose	mg	% of inj dose	
33	17	27	13	6 87	66	0 60	5 8	8 0
44	29	20	13	5 48	57	0 51	5 3	8 5
17	20	9	8	1 63	42	0 26	6 7	14
13	12	10	12	1 54	42	0 24	6 5	13
8	8	<3	7	0 87	44	0 12	6 0	12
<3	8	<3	3	0 76	42	0 09	5 0	11
16	14	<9	13	0 62	31	0 19	10	23

The *nor*morphine excreted in the urine amounted to 6–7% of the morphine injected and there seemed to be no relationship between percentage excretion and the amount of morphine injected Free *nor*morphine amounted to 9% of total *nor*morphine after injection of 20 mg/kg morphine The joint excretion of morphine and *nor*morphine in the urine amounted to 40–70% of the morphine injected

The *nor*morphine excreted in the urine from the single daily specimens, as a percentage of the total urinary excretion of both *nor*morphine and morphine are given in fig 1 *Nor*morphine only accounts for about 10% of the total amount excreted on the first day but the percentage increased each day with the most marked increase for the small doses Five days after injection of 10 mg/kg only *nor*morphine was found in the urine

The faecal excretion of *nor*morphine and morphine after subcutaneously injected morphine is given in table 2 In the faeces 7–10% of the morphine injected was found as morphine and 2–3% as *nor*morphine After injection of 20 mg/kg morphine, about 60% of the total amount of morphine in the faeces was unconjugated, and all the demonstrable *nor*morphine was unconjugated An error may arise from the fact that a small part of the conjugated drug is hydrolysed when the faeces are suspended *in N HCl for 24 hours (see methods)* The average of the total excretion of morphine and *nor*morphine in the faeces amounted to about 10% of the injected morphine

Discussion

The elimination of morphine in the urine and faeces of rats given morphine has been investigated quantitatively by FICHTENBERG (1951) She found 48% of the injected dose in the urine and 12% in the faeces after subcutaneous injection of 200 mg/kg These figures indicate a

Table 1

Excretion of morphine and *nor*morphine in the

Groups of animals no	Weight g (mean*)	Dose of morphine mg/kg	Excretion of morphine and									
			Time after									
			0–6 hrs		6–24 hrs		1 day		2 day		3 day	
			M µg	N µg	M µg	N µg	M µg	N µg	M µg	N µg	M µg	N µg
1	208	50	–	–	–	–	6100	460	610	83	100	24
2	192	50	530	15	4030	330	4560	350	710	78	150	37
3	193	20	610	72	710	98	1320	170	230	35	49	25
4	185	20	830	77	440	85	1270	160	170	31	73	25
5	202	10	460	47	320	40	780	87	72	13	13	9
6	180	10	450	32	220	23	670	55	80	12	13	8
7	204	10	320	30	190	33	510	63	70	54	23	4?

* All figures are averages for 3 pooled rats

Free morphine and *nor*morphine were determined in the urine of rats in group 4 Free morphine amounts to 27% of total morphine and free *nor*morphine to 9% of total *nor* morphine

M = morphine
N – *nor*morphine

Each specimen was analysed for morphine and *nor*morphine by the method employed by MILTHERS (1961) for tissues with the following modification The paper used for the chromatography was buffered at pH 7 0, permitting morphine and *nor*morphine to be separated over a 16 hours period For determining morphine and *nor*morphine as conjugated (free) compounds the urine was extracted directly with the organic solvents The faeces was suspended in N-HCl for 24 hours at 4°C and filtered and the filtrate was then extracted with the organic solvents When determining in bulk free and conjugated morphine, or *nor*morphine the urine was first autoclaved after addition of 10% 10 N-HCl before extraction The faeces was suspended in N-HCl and autoclaved before filtration and extraction Recovery experiments with urine and faeces showed the same percentage recovery as was found by MILTHERS (1961) for blood and tissues (That is, for morphine about 96%, for *nor*morphine 72%) In calculating the *nor*morphine values allowance was made for the low percentage recovery and the values were converted to morphine Control urine and faeces were sampled before treatment and yielded no blank values

Results

The urinary excretion of *nor*morphine and morphine after morphine injected subcutaneously is given in table 1 Of the injected morphine 40–65% could be accounted for in the urine The results show a relationship between percentage excretion and the amount injected The lowest percentage excretion is found for the smallest doses Thus, after the injection of 10 mg/kg, 31–44% was excreted, after 20 mg/kg 42%, and after 50 mg/kg, 57–66% Free morphine amounted to 27% of the total morphine after 20 mg/kg

Table 2.

Excretion of morphine and *nor*morphine in the faeces of rats after subcutaneous morphine

Group no	Weight g (mean*)	Dose of morphine mg/kg	Excretion of morphine and *nor*morphine in faeces												Excreted *nor*morphine as per cent of excreted *nor*morphine + morphine
			Time after injection								Total				
			1 day		2 day		3 and 4 day		5 day		morphine		*nor*morphine		
			M µg	N µg	M µg	N µg	M µg	N µg	M µg	N µg	µg	% of inj dose	µg	% of inj dose	
1	208	50	252	22	346	126	24	32	<5	<8	622	6.0	180	1.7	22
2	192	50	286	69	148	55	236	90	50	46	720	7.4	260	2.7	27
3	193	20	69	17	170	66	22	23	<4	<7	261	6.7	106	2.7	30
4	185	[illegible]	pooled with 2 day		357	102	15	17	<4	<7	372	10	119	3.2	24
5	202	10	[illegible]	16	41	17	16	10	–	–	137	6.9	43	2.2	24
6	180	10	57	10	123	23	7	8		–	180	10	33	1.8	15
7	204	10	27	8	114	29	50	[illegible]	<3	–	191	9.6	29	1.5	17

* All figures are averages for 3 pooled rats

Free *nor*morphine and morphine were determined in the faeces of rats in group 4 Free morphine amounted to 61% of total morphine and free *normorphine to* 100% *of total nor*morphine

M = morphine
N – *normorphine*

We found total urinary recovery of both free and conjugated morphine to be increased by an increased supply of morphine, whereas the total percentage of *nor*morphine excreted in the urine was independent of the amount of morphine given This divergence is understandable, if it is assumed that only free morphine is biologically transformed to *nor*morphine, because PÆRREGAARD (1958 a) has shown that the percentage of *free* morphine excreted in the urine is independent of the amount of morphine given

*Nor*morphine, like morphine, was predominantly excreted in the urine during the first 24 hours In the first 24 hours specimen *nor*morphine accounted for 60–70% of the total excretion of *normorphine*, and morphine for 80–90% of the total excretion of morphine The urinary excretion of *normorphine* was more prolonged than that of morphine Five days after injection of the smallest dose the urine only contained *nor*morphine This is in accordance with the results of FRASER *et al* (1958), who found a markedly cumulative effect of *nor*morphine after multiple doses of the drug, indicating that *nor*morphine was *retained* by the tissues for a fairly long period

In our experiments we found that the total *excretion* of *nor*morphine in

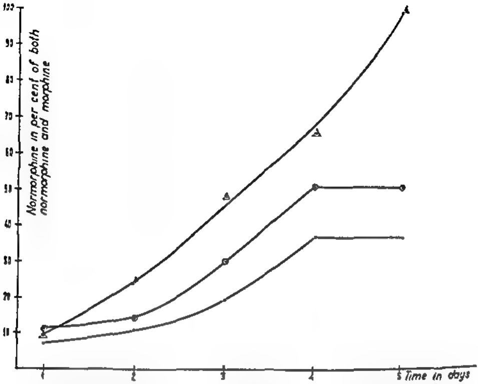

Fig 1 The *nor*morphine excretion in urine from the single daily specimens, as percentage of the total urinary excretion of both *nor*morphine and morphine From rats injected subcutaneaously with 10 mg/kg △———△, 20 mg/kg ⊚———⊚, and 50 mg/kg •———• morphine

somewhat lower excretion in the urine and a somewhat higher excretion in the faeces than were found in our experiments

The excretions in the urine of dogs and in the urine and faeces of monkeys after subcutaneously injected *nor*morphine have recently been investigated by MISRA, JACOBY & WOODS (1961 a) In monkeys they found the urinary recovery to be 70–80% of the injected dose (2 mg/kg) Free *nor*morphine accounted for about one quarter In the faeces they found 20–40% of the injected dose, and only a small percentage of this was conjugated

In agreement with these authors we were able to demonstrate only the unconjugated form of *nor*morphine in the faeces When the amounts of *nor*morphine and morphine in the faeces are related to their total amounts eliminated in faeces plus urine, higher values were found for *nor*morphine than for morphine This is also in agreement with the authors mentioned above However, we found only a tenth of the *nor*morphine excreted in the urine as *free nor*morphine Thus there seems to occur a more extensive conjugation of biologically formed *nor*morphine than of injected *nor*morphine This is what happens with morphine biologically transformed from codeine (PÆRREGAARD 1958 b)

Table 2.

Excretion of morphine and *nor*morphine in the faeces of rats after subcutaneous morphine.

Group no	Weight g (mean*)	Dose of morphine mg/kg	Excretion of morphine and *nor*morphine in faeces												Excreted *nor*morphine as per cent of excreted *nor*morphine + morphine
			Time after injection								Total				
			1 day		2. day		3 and 4 day		5 day		morphine		*nor*morphine		
			M µg	N µg	M µg	N µg	M µg	N µg	M µg	N µg	µg	% of inj dose	µg	% of inj dose	
1	208	50	252	22	346	126	24	32	<5	<8	622	6.0	180	1.7	22
2	192	50	286	69	145	55	236	90	50	46	720	7.4	260	2.7	27
3	193	20	69	17	170	66	22	23	<4	<7	261	6.7	106	2.7	30
4	185	20	pooled with 2. day		357	102	15	17	<4	<7	372	10	119	3.2	24
5	202	10	80	16	41	17	16	10	–	–	137	6.9	43	2.2	24
6	180	10	37	10	123	23	7	8	–	–	180	10	33	1.8	15
7	204	10	27	8	114	29	50	10	<3	–	191	9.6	29	1.5	17

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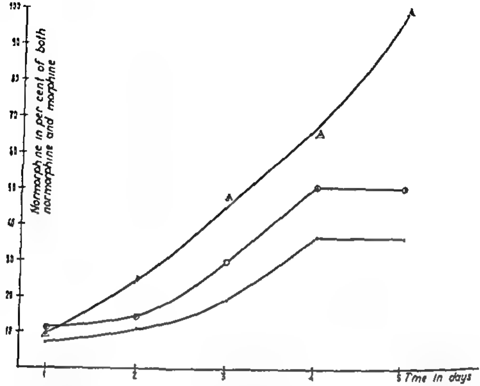

Fig 1 The *nor*morphine excretion in urine from the single daily specimens as percentage of the total urinary excretion of both *nor*morphine and morphine From rats injected subcutaneaously with 10 mg/kg △——△, 20 mg/kg ○——○, and 50 mg/kg ●——● morphine

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Group no	Weight g (mean*)	Dose of morphine mg/kg	Excretion of morphine and *nor*morphine in faeces: Time after injection: 1 day M μg	1 day N μg	2. day M μg	2. day N μg	3 and 4 day M μg	3 and 4 day N μg	5 day M μg	5 day N μg	Total: morphine μg	morphine % of inj dose	*nor*morphine μg	*nor*morphine % of inj dose	Excreted *nor*morphine as per cent of excreted *nor*morphine + morphine
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6	180	10	57	10	123	23	7	8		–	180	10	33	1 8	15
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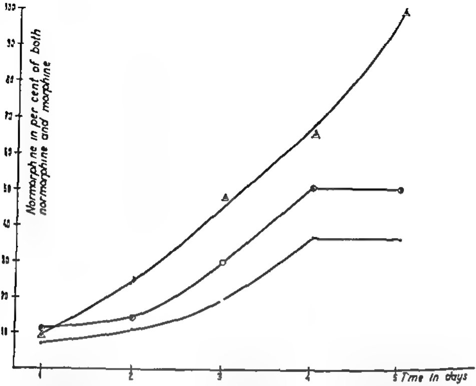

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Milthers Kirsten Normorphine, nalorphine and morphine Quantitative separation and determination of small amounts in blood and tissues *Acta pharmacol et toxicol* 1961, [illegible] 199–206
Misra A L, H I Jacoby & L A Woods The preparation of tritium labeled normorphine and its physiological disposition in dog and monkey *J Pharmacol* 1961 a 132 311–316
Misra A L, S J Mulé & L A Woods The preparation of tritium nuclear labelled morphine and evidence for its in vivo biotransformation to [illegible]

Pærregaard P The liberation of morphine from codeine in man and dog *Acta pharmacol et toxicol* 1958 b 14 394–399

urine and faeces ammounted to 8–9% of the dose of morphine given, indicating that at least 8–9% of the morphine given is N-demethylated in rats. In monkeys MISRA *et al.* (1961 a) found that all the *nor*morphine given was found in the urine and faeces, but this is probably not true of rats. With morphine the urinary recovery is 70–80% in monkeys (MELLET & WOODS 1956), but only 40–50% in rats. MISRA and his collaborators also found the urinary recovery of *nor*morphine in monkeys to be 70–80%. If the same relationship is valid for *nor*morphine, we can estimate the degree of N-demethylation of morphine in rats. We found in the urine about 6% of the administered morphine as *nor*morphine. This is therefore the amount responsible for 40–50% of the available *nor*morphine, and about 13% of the morphine given must have been N-demethylated in rats to agree with our finding that the small doses caused excreted *nor*morphine to account for 12–13% of the urinary excretion of both *nor*morphine and morphine.

Summary

The urinary and faecal excretions of *nor*morphine and morphine after increasing doses of morphine injected subcutaneously into rats have been studied.

1. The recoveries of morphine in urine and faeces were 40–50% and 7–10%, respectively. The relative urinary excretion increased with increasing dose of morphine administered.

2. The amounts of *nor*morphine in urine and faeces were 6–7% and 2–3%, respectively, independently of the dose of morphine.

3. It is concluded that 12–13% of subcutaneously injected morphine is transformed to *nor*morphine in rats.

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Summary

The urinary and faecal excretions of *nor*morphine and morphine after increasing doses of morphine injected subcutaneously into rats have been studied

1 The recoveries of morphine in urine and faeces were 40–50% and 7–10%, respectively The relative urinary excretion increased with increasing dose of morphine administered

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Fraser H F A Wikler G D van Horn A J Eisenman & H Isbell Human pharmacology and add ction liability of normorphine *J Pharmacol* 1958 122 359 369

March C H & H W Elliott Distribution and excretion of rad oactivity [illegible] C^{14} to rats *Proc Soc Exper*

[illegible] nd fate of morphine in the [illegible] *acol* 1956 116 77 83

Material and Methods

Fifty six rabbits (26 males, 30 females) weighing between 2 3–2 9 kg, were used for the experiments

Normal rabbits

As a control series for determining the catalase activity of the liver, 5 animals were used (2 males, 3 females) The concentration of green porphyrins in the liver was determined in 19 animals (10 males, 9 females) The 24-hour outputs of δ-aminolaevulic acid (ALA), porphobilinogen (PBG) and coproporphyrin (CP) were measured in 50 animals (25 males, 25 females) and that of uroporphyrin (UP) in 40 animals (15 males, 25 females) The 24-hour faecal outputs of CP and protoporphyrin (PP) were determined in 54 rabbits (26 males, 28 females)

Apronal-intoxicated rabbits

Nine rabbits (2 males, 7 females) were given apronal in doses of 100–400 mg/kg bodyweight (b w) The distribution of the doses is given in fig 1 The substance was dissolved in 10 ml of 96% ethyl alcohol and given by stomach tube Immediately afterwards the animals received about 20 ml of water by the same route Four of the animals (Nos 6, 7, 8, 9) were placed in metabolism cages after administering the apronal The 24-hour samples of urine and faeces were collected separately. The excretion of ALA, PBG, UP and CP in the urine and of CP and PP in the faeces was determined All 9 animals were killed the day after the last dose of apronal, and the livers were removed for determining the catalase activity and the concentration of green porphyrins

DDTD-intoxicated rabbits

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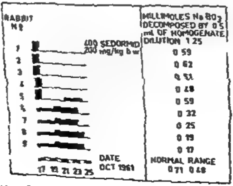

Fig 1 Doses of apronal (sedormid ®) given to 9 rabbits The catalase activity of the liver was determined the day after the last dose

Acta pharmacol et toxicol 1962, 19, 156–164

From the Department of Clinical Chemistry, University of Lund Malmo General Hospital, Malmo Sweden (Ass Professor C-B Laurell M D)

Porphyria Induced in the Rabbit by Diethyl 1,4-Dihydro-2,4,6-Trimethylpyridine-3,5-Dicarboxylate

II Catalase activity and concentration of green porphyrins in the liver and a comparison with apronal induced porphyria

By

Birgitta Haeger-Aronsen

(Received May 3 1962)

A type of hepatic porphyria induced by oral administration of diethyl 1,4 dihydro 2,4,6 trimethylpyridine-3,5 dicarboxylate (DDTD) in the rabbit was described in an earlier paper (HAEGER-ARONSEN 1961)

The two previously known types of experimental hepatic porphyria are induced by derivatives of α substituted allylacetic acid, usually *allyliso* propylacetylcarbamide[1]) (SCHMID & SCHWARTZ 1952, BENARD *et al* 1953 GOLDBERG 1954, and others), and by hexachlorobenzene (SCHMID 1960 OCKNER & SCHMID 1961, DE MATTEIS *et al* 1961)

All three types of porphyria are characterised by an increased excretion of porphyrins and their precursors

It has been shown that the catalase activity of the liver in apronal intoxicated rats and rabbits is significantly decreased and that the decrease is due to inhibited synthesis of this enzyme (SCHMID & SCHWARTZ 1952, 1955, SCHMID *et al* 1953, SCHMID *et al* 1955) It has however not been possible to demonstrate whether apronal also accelerates the breakdown of liver catalase The activity of catalase in the erythrocytes like that of cytochrome C and cytochrome oxidase in the liver, is not affected by apronal (SCHMID *et al* 1955) Increased amounts of "green porphyrins – an ill defined type of pigments – have been demonstrated in the liver during experimental apronal intoxication (SCHWARTZ 1955, SCHWARTZ & IKEDA 1955)

OCKNER & SCHMID (1961) found the catalase activity of the liver in hexachlorobenzene poisoned rats to be normal The metabolic block induced by this type of intoxication is not fully understood

The main purpose of this investigation was to ascertain whether the catalase activity and the concentration of green porphyrins in the liver undergo any changes associated with DDTD intoxication

1) Sedormid ® = apronal (NFN)

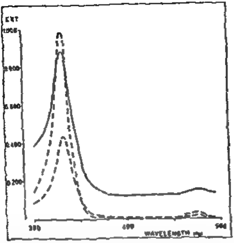

Fig 3 Absorption curves for green porphyrins
- - - Extracted from livers of apronal intoxicated rabbits ——— Extracted from liver of a DDTD intoxicated rabbit Maxima found at 413 and 553 mμ

Results

Normal values

The results of determining the catalase activities of the livers in 5 healthy rabbits are given in fig 4A The number of millimoles of $NaBO_3$ decomposed by 0 5 ml of the 4% homogenates ranged between 0 71–0 48

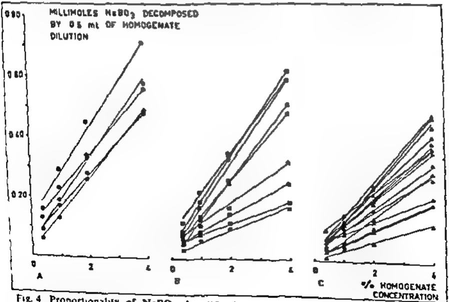

Fig. 4 Proportionality of $NaBO_3$ decomposition to concentration of enzyme source A 5 normal rabbits B 9 apronal intoxicated rabbits C 15 DDTD intoxicated rabbits

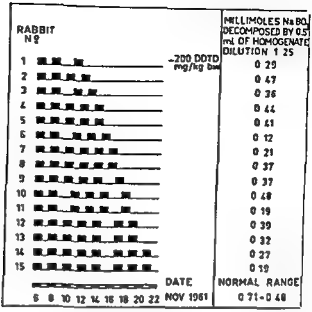

Fig 2 Doses of DDTD given to 15 rabbits The catalase activity of the liver was determined the day after the last dose

moved for measurement of the catalase activity and the concentration of green porphyrins

Control animals

Four animals (1 male, 3 females) received 10 ml of 96% ethyl alcohol each and then about 20 ml of water by stomach tube This dose was given every other day for 18 days On the day after the last dose the animals were killed and the livers removed for determining catalase activity and concentration of green porphyrins

Immediately after removal, the liver slices used for determining catalase activity were washed and homogenised, as described by DOUNCE & SHANEWISE (1950) The mean wet weight per millilitre of the homogenates was 0 97 g (0 91–1 02) and the corresponding mean dry weight 0 073 g (0 021–0 100) For determining the catalase activity each homogenate was diluted 1 25 (4%), 1 50 (2%) 1 100 (1%) and 1 250 (0 4%) with distilled water

The *catalase activity* in the various dilutions was determined by the method of FEINSTEIN (1949) As for the controls, each dilution was analysed twice, and the mean was calculated The activity was expressed as millimoles of sodium perborate ($NaBO_3$) decomposed by 0 5 ml of homogenate dilution under the conditions of the method used The calculations were made after correcting the wet weights of the original homogenates to 1 00 g per ml

Green porphyrins were determined by the method of SCHWARTZ & IKEDA (1955) The pigments were extracted from 10 g of liver The livers were kept in the dark at +4°C until they were analysed, which was always at most 6 hours after the death of the animals The extinctions of the 7 5 N HCl extracts were read at 413 mμ, since the "Soret maximum" was found to be situated there in more than 80% of the specimens (fig 3) The determinations were made in a Beckman spectrophotometer, model DU, in a 1 cm cuvette against a blank consisting of 7 5 N HCl

Other methods used have been described previously (HAEGER 1958, HAEGER-ARONSEN 1960, 1961)

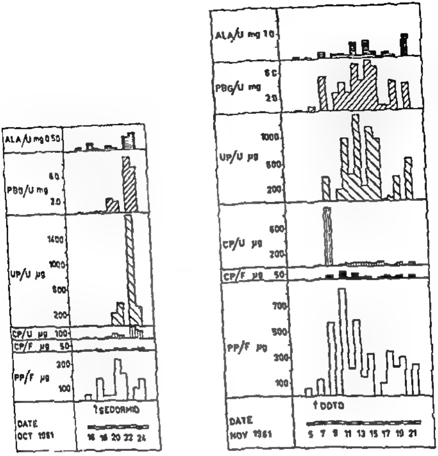

Fig 6 Mean urinary 24-hour excretion of δ-aminolaevulic acid (ALA), porphobilinogen (PBG) uroporphyrin (UP) and coproporphyrin (CP) and also of faecal CP and protoporphyrin (PP) by 15 DDTD intoxicated rabbits – Arrow indicates first day of DDTD administration

the animals The increase was most marked in those animals receiving the largest doses of apronal In these it was 10–20 times the normal value

The individual curves for the excretion of ALA, PBG, UP and CP in the urine and of CP and PP in the faeces in the 4 animals studied (Nos 6, 7, 8 9) were similar in shape. The mean values for the group are given in fig 5

Table 1.

Normal urinary excretion (w/24 hrs) of δ-aminolaevulic acid (ALA) porphobilinogen (PBG), uroporphyrin (UP) and coproporphyrin (CP) by rabbits

Substance	Number of determinations	w/24 hrs	m	S D	95% range
ALA	50	mg	0 08	0 09	0 00–0 26
PBG	50	mg	0 07	0 08	0 00–0 23
UP	40	μg	0	0	–
CP	50	μg	5	5	0–15

Table 2.

Normal faecal excretion (μg/24 hrs) of coproporphyrin (CP) and protoporphyrin (PP) by rabbits

Substance	m	S D	95% range
CP	4	7	0–18
PP	53	33	0–119

Table 3

Hepatic catalase activity and concentration of green porphyrins in animals with experimental porphyria
N – normal D – decreased, I – increased,
– – *information not available*

Type of porphyria	Catalase activity	Concentration of green porphyrins
Apronal induced (rats rabbits)	D	I
Hexachlorbenzene induced (rats)	N	–
DDTD induced		
(rats)	–	N
(rabbits)	D	I

Green porphyrins from 19 rabbits gave an extinction of 0 3–1 1 per 10 g of liver

The urinary excretion values for ALA, PBG, UP and CP are given in table 1 and those for the faecal excretion of CP and PP in table 2

Apronal-intoxicated rabbits

The values found for the catalase activity of the livers of 9 apronal-intoxicated rabbits are given in fig 4B, which shows that the activity was normal in 5 animals but appreciably reduced in the remaining 4

The concentration of green porphyrins in the liver was increased in all

& FIGGE's (1960) finding of a normal concentration of the green porphyrins in the liver in DDTD intoxicated rats can probably be explained by the difference in the animal species

The excretion of ALA, PBG, UP and CP in the urine was increased in intoxication with apronal and DDTD STICH (1958) has reported the ALA excretion to be normal in apronal intoxicated rabbits, but ABBOTT & RUDOLPH (1961) found a pathological excretion of this metabolite by rabbits and rats intoxicated in this way The CP excretion in the faeces was only moderately increased in apronal as well as in DDTD intoxication and the excretion of PP was clearly elevated in both types

The observations made are compatible with the assumption that essentially the same type of metabolic block occurs in apronal- and DDTD intoxication In the production of experimental porphyria, it would appear that DDTD is to be preferred to apronal, because it produces a much lower mortality

Summary

In porphyria induced by oral administration of diethyl 1,4-dihydro-2 4 6-*trimethyl*pyridine-3,5-dicarboxylate (DDTD) or allyl*iso*propylacetylcarbamide (apronal) to rabbits, the liver showed a substantially reduced catalase activity and an increased concentration of green porphyrins

The excretions of δ aminolaevulic acid (ALA), porphobilinogen (PBG), uroporphyrin (UP) and coproporphyrin (CP) in the urine, as well as of CP and protoporphyrin (PP) in the faeces, were increased in both types of intoxication

In view of these biochemical similarities the metabolic block induced is assumed to be the same in the two types of porphyria

Acknowledgements

I thank Mrs Mariann Nilsson and Miss Kerstin Delbrant for technical assistance The investigation was supported by grants from the Swedish Medical Research Council

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DDTD-intoxicated rabbits

The values found for the catalase activity of the livers of 15 DDTD intoxicated rabbits are given in fig 4C, which shows that the activity was reduced in all of the animals

The concentration of green porphyrins *in the liver* was invariably increased to 10–50 times the normal value

The individual curves for the urinary excretion of ALA, PBG, UP and CP and of faecal CP and PP were similar The mean values for the group are given in fig 6

Control animals

The catalase activity of the livers from the 4 control animals, which received ethyl alcohol only, ranged between 0 68 and 0 52 millimoles of $NaBO_3$ decomposed by 0 5 ml of 4% homogenate, *i e* the values were normal

The concentration of green porphyrins in the livers of these animals was normal

Discussion

The catalase activity of the liver was normal in the 5 animals receiving relatively small doses of apronal (400–700 mg/kg b w), but decreased in the other 4 animals that had received larger doses (1100–1300 mg/kg b w) This confirms the observation of previous workers that the catalase activity of the liver of rabbits is decreased after administering large amounts of apronal (SCHMID & SCHWARTZ 1952, 1955, SCHMID *et al* 1953, SCHMID *et al* 1955)

In the animals that had received DDTD, the liver catalase activity also decreased The decrease was not proportional to the DDTD dose The dose of alcohol used in these experiments was large, so that possibly the reduction of the activity may have been due to alcohol-induced changes in the liver However this appears unlikely, since the activity was normal in the controls receiving alcohol only The possibility that alcohol produces some effect when used in combination with DDTD cannot, however, be excluded If DDTD alone is responsible for the reduction it might be due to (1) Inhibition of the enzyme synthesis, by analogy with what is seen in apronal intoxication, (2) Increased breakdown of liver catalase, or (3) Inhibited catalytic effect of the enzyme

SCHWARTZ (1955) found the concentration of green porphyrins in the liver to be increased *in apronal-intoxicated rabbits* Our findings agree with this The DDTD intoxicated animals also showed a substantially increased concentration of these porphyrins in the liver SOLOMON

Acta pharmacol et toxicol 1962, 19, 165–180

From the Department of Pharmacology, University of Goteborg
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The Effects of Hydralazine Administered into the Brachial Artery on Adrenergic Vasoconstrictor Stimuli in the Hand

By

Åblad, B, G Johnsson and M Henning

(Received May 12 1962)

The hypotensive effect of hydralazine is due to a decrease in vascular resistance (MOYER *et al* 1951, WILKINSON *et al* 1952, FREIS *et al* 1953 and others) Studies on animals have given rise to divergent opinions as to whether the site of action is central or peripheral (cf GROSS *et al* 1950, CRAVER *et al* 1951, GRIMSON *et al* 1952, BEIN *et al* 1953, LIM *et al* 1955, SCHMITT & GICQUEL 1956, KIRPEKAR & LEWIS 1957, TANGRI & BARGHAVA 1960) In man the peripheral point of action seems to be predominantly important for the decrease in vascular resistance (STUNKARD *et al* 1954, ÅBLAD 1959)

In a recent investigation (ÅBLAD *et al* 1961) we found that hydralazine administered into a brachial artery produced a substantial rise in blood flow through the proximal part of the forearm and the hand, whereas the blood flow in the opposite arm as well as blood pressure and pulse rate were unaffected The dose was such that the drug's concentration in the arm was probably equivalent to that obtained by systemic administration of therapeutic doses The local vasodilatation developed slowly and persisted for more than one hour, in the same way as the hypotensive effect after intravenously administered hydralazine

To analyze further the peripheral action, we have investigated the influence of intra arterially administered hydralazine on vasoconstriction in the hand as induced by a cold test or by infusion of noradrenaline In one series of experiments hydralazine was given in a higher dose of 0 95–1 27 mg, and in another series a lower dose of 0 30–0 55 mg (similar to that used in the above mentioned study)

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further influenced by some humoral factor released from the adrenals, probably adrenaline – In the results reported below, the effect of the cold test on blood flow in the hand is recorded both as values observed 10 seconds after the application of the stimulus and as those recorded after 40 and 60 seconds

The drugs used were hydralazine (1 hydrazinophtalazine chloride, apresoline ®, Ciba) and noradrenaline (1 noradrenaline bitartrate, Noradrenalin ®, Byk Gulden) The infused noradrenaline solutions contained as a preservative 0 001 per cent ascorbic acid (GADDUM *et al* 1949)

The statistical analysis was based on the *t* test (FISHER 1958) Results are reported as means ± standard error of the mean In statistical analysis of the difference in blood flow between two measuring periods (before and after administration of an agent) the absolute changes of flow in the individual experiments were used as variates

Results

A Effects of Intra arterially Administered Hydralazine on the Blood Flow

After administration of hydralazine in one brachial artery, the blood flow in the ipsilateral hand (test hand) increased, and this effect persisted throughout the entire experiment in all subjects The blood flow in the contralateral hand (control hand) showed varying changes in different experiments, though the average changes were insignificant The differences between the blood flows in the hydralazine treated test hand and in the control hand were, on an average, approximately the same before application of the first as before application of the last vasoconstrictor stimulus

1 Effect of the Higher Dose of Hydralazine

Calculation of the influence of hydralazine on the blood flow recorded before the application of vasoconstrictor stimuli provided an estimate of the vasodilator effect of the drug In the seven experiments with the higher dose [illegible] administration of hydralazine the test hand blood flow increased to an average of 36 6 ± 3 72 ml The increase of blood flow was *statistically significant* (*difference 16 3 ± 3 15 ml*, $P < 0\ 01$) The simultaneously recorded blood flow values in the control hand were 19 8 ± 2 20 ml before and 19 5 ± 1 89 ml after the administration of hydralazine (mean decrease 0 3 ± 1 05 ml)

2 Effect of the Lower Dose of Hydralazine

In the ten experiments with the lower dose of hydralazine, the mean blood flow in the test hand increased from 16 5 ± 2 69 ml to 25 2 ± 2 34

Methods

The experiments were performed on healthy students The recumbent subject was covered with a blanket and the experiments were performed at a room temperature of 27 ± 1°C Under these conditions the subject felt comfortably warm without sweating A thin polyethylene or teflon catheter was introduced into the brachial artery as previously described (ÅBLAD *et al* 1961), and through it drugs or isotonic saline were infused at a constant rate (in most experiments 0 6 ml per minute) from a motor driven syringe Noradrenaline was infused into a superficial vein of the arm from another motor-driven syringe Both hands were placed in plethysmographs filled with water at a temperature of 33°C The experiments were performed with the subject's arms at their sides As a rule the capacity of the venous reservoir in the hand was then large enough to give measurable inflow curves even at high bloodflow levels (*cf* RODDIE & SHEPHARD 1956) Other details of the plethysmographic method used have been already described (ÅBLAD *et al* 1961)

The blood flow in the hand is characterized by pronounced spontaneous variations and is greatly influenced by emotional stimuli The subjects were therefore instructed to relax and were not aware of the exact time of drug infusions It has been shown that the spontaneous variations are similar in the two hands (COOPER *et al* 1950) and, since in all experiments the blood flow was determined simultaneously in both hands it was possible to distinguish these variations from the local unilateral effects of the intra arterially administered drugs

Each experiment proceeded according to a general pattern Initially a study was made of the effects of a cold test and of an intravenous or intra arterial infusion of noradrenaline or both For the cold test a piece of ice was placed in the subject's mouth for 70 seconds Noradrenaline was infused for five minutes, occasionally for somewhat longer, and was given intravenously in a dose of 12–24 µg per minute or intra arterially in a dose of 0 15–0 42 µg per minute During the cold test the blood flow was determined by means of venous occlusions after 10, 40, and 60 seconds otherwise venous occlusion was generally produced simultaneously in both arms once or twice per minute The different vasoconstrictor stimuli did not follow the same sequence in all experiments The intervals between stimuli ranged from 7 to 20 minutes, and the next stimulus was not applied until the effect of the previous stimulus was judged to have subsided completely – After these tests hydralazine was infused intra arterially over a five-minute period, the higher dose (0 95–1 27 mg) being given in seven experiments and the lower dose (0 30–0 55 mg) in ten others Thirty minutes later when the hydralazine effect was relatively constant (ÅBLAD *et al* 1961) the tested vasoconstrictor stimuli were again applied in the same order and dosage, and with the same duration, as before In three subjects in whom the effects of the cold test and of intravenously infused noradrenaline were studied the experiments were repeated at a later date when we recorded the arterial blood pressure, measured by the method of RIVA-ROCCI, instead of the blood flow in the hands

... ents made

... s

of determinations made during the last two minutes of the infusion

In the cold test, the blood flow in the hand (BARCROFT & SWAN 1953) and the fore-arm (WILKINS & EICHNA 1941) falls after a latent period of a few seconds According to WILKINS & EICHNA, the initial effect in the forearm is due solely to increased activity in the vasoconstrictor fibres, but after 30 seconds the forearm circulation is sometimes

Table I

Effect of the cold test on blood flow in the hands before and after intra arterial administration of hydralazine in the higher dose range. Blood flow expressed as ml per 100 ml tissue per minute

Exp No	Hand volume ml		Intra arterial dose of hydralazine mg	Blood flow											
				Before hydralazine						After hydralazine					
				Basal		Cold test				Basal		Cold test			
						10 seconds		40 60 sec				10 seconds		40, 60 sec	
	Test hand	Contr hand		Test hand	Contr hand	Test hand	Contr hand	Test hand	Contr hand	Test hand	Contr hand	Test hand	Contr hand	Test hand	Contr hand
11	465	475	0.95	27.9	25.4	7.2	5.7	9.2	8.7	31.2	20.1	20.1	7.6	22.6	8.8
12	370	400	1.11	9.2	11.1	3.6	3.1	7.6	6.9	26.2	20.4	15.8	4.0	23.4	8.9
13	340	355	1.11	12.9	12.8	1.5	2.0	2.9	2.8	48.1	22.5	23.5	4.1	23.4	7.3
14	400	355	1.11	14.4	12.1	12.1	8.7	9.1	8.6	21.5	13.2	17.9	11.4	17.9	10.8
15	415	420	1.27	18.6	19.4	11.8	11.1	8.1	8.0	43.4	13.8	42.7	6.3	50.1	7.5
Mean value s e m	398 ±21.1	401 ±22.4	1.11 ±0.051	16.6 ±3.20	16.1 ±2.73	7.2 ±2.13	6.1 ±1.70	7.4 ±1.16	7.0 ±1.10	34.5 ±5.26	18.0 ±1.89	24.0 ±4.85	6.7 ±1.36	27.5 ±5.75	8.7 ±0.63

ml The change amounted to 8 7 ± 1 39 ml ($P < 0\,001$) The simultaneously measured blood flow in the test hand decreased insignificantly from 17 0 ± 2 85 ml to 16 6 ± 2 45 ml (difference 0 4 ± 1 22 ml) – After the higher dose of hydralazine the mean increase of blood flow in the test hand was 7 6 ± 3 44 ml greater than that after the lower dose ($P < 0\,05$)

B Effect of Hydralazine on the Vasoconstrictor Action of the Cold Test in the Hand

The cold test had little effect on the blood pressure In the three experiments in which this factor was studied, the systolic and diastolic blood pressure increased by 0–10 mm Hg The changes occurring before and after intra-arterial administration of hydralazine were of equal magnitude

1 Effect of the Higher Dose of Hydralazine

The effect of the cold test on the blood flow in the hands was studied in five experiments before and after administering the higher dose of hydralazine (table 1, figure 1) Before the infusion of hydralazine the cold test produced a quantitatively similar reduction of blood flow in the two hands After 10 seconds the mean decrease was 9 4 ± 3 19 ml in the test hand and 10 0 ± 2 69 ml in the control hand (difference 0 6 ± 0 64 ml), after 40 and 60 seconds, 9 3 ± 2 88 ml in the test hand and 9 2 ± 2 44 ml in the control hand (difference 0 1 ± 0 86 ml) After the administration of

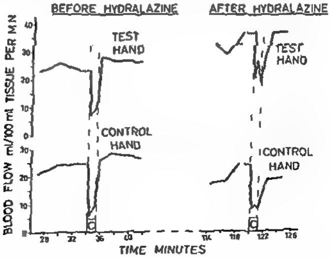

Fig 1 Effect of the cold test on blood flow in the hands before and after intra arterial administration of hydralazine Cold test performed at 34th and 120th minute Hydralazine (0 95 mg) given at 67th–72nd minute

Table I

Effect of the cold test on blood flow in the hands before and after intra arterial administration of hydralazine in the higher dose range Blood flow expressed as ml per 100 ml tissue per minute

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11	465	475	0 95	27 9	25 4	7 2	5 7	9 2	8 7	31 2	20.1	20 1	7 6	22 6	8 8
12	370	400	1 11	9 2	11 1	3 6	3 1	7 6	6 9	26 2	20 4	15 8	4 0	23 4	8 9
13	340	355	1 11	12 9	12 8	1 5	2 0	2 9	2 8	48 1	22 5	23 5	4 1	23 4	7 3
14	400	355	1 11	14 4	12 1	12 1	8 7	9 1	8 6	21 5	13 2	17 9	11 4	17 9	10 8
15	415	420	1 27	18 6	19 4	11 8	11 1	8 1	8 0	45 4	13 8	42 7	6 3	50 1	7 5
Mean value s e m	398 ±21 1	401 ±22 4	1 11 ±0 051	16 6 ±3 20	16 1 ±2 73	7 2 ±2 13	6 1 ±1 70	7 4 ±1 16	7 0 ±1 10	34 5 ±5 26	18 0 ±1 89	24 0 ±4 85	6 7 ±1 36	27 5 ±5 75	8 7 ±0 63

ml The change amounted to 8 7 ± 1 39 ml (P < 0 001) The simultaneously measured blood flow in the test hand decreased insignificantly from 17 0 ± 2 85 ml to 16 6 ± 2 45 ml (difference 0 4 ± 1 22 ml) - After the higher dose of hydralazine the mean increase of blood flow in the test hand was 7 6 ± 3 44 ml greater than that after the lower dose (P < 0 05)

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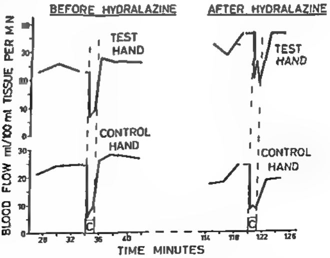

Fig 1 Effect of the cold test on blood flow in the hands before and after intra arterial administration of hydralazine Cold test performed at 34th and 120th minute Hydralazine (0 95 mg) given at 67th 72nd minute

Table 1

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Exp No	Hand volume ml		Intra arterial dose of hydralazine mg	Blood flow											
				Before hydralazine						After hydralazine					
				Basal		Cold test				Basal		Cold test			
						10 seconds		40–60 sec				10 seconds		40–60 sec	
	Test hand	Contr hand		Test hand	Contr hand	Test hand	Contr hand	Test hand	Contr hand	Test hand	Contr hand	Test hand	Contr hand	Test hand	Contr hand
11	465	475	0.95	27.9	25.4	7.2	5.7	9.2	8.7	31.2	20.1	20.1	7.6	22.6	8.8
12	370	400	1.11	9.2	11.1	3.6	3.1	7.6	6.9	26.2	20.4	15.8	4.0	23.4	8.9
13	340	355	1.11	12.9	12.8	1.5	2.0	2.9	2.8	48.1	22.5	23.5	4.1	23.4	7.3
14	400	355	1.11	14.4	12.1	12.1	8.7	9.1	8.6	21.5	13.2	17.9	11.4	17.9	10.8
15	415	420	1.27	18.6	19.4	11.8	11.1	8.1	8.0	45.4	13.8	42.7	6.3	50.1	7.5
Mean value s.e.m.	398 ±21.1	401 ±22.4	1.11 ±0.051	16.6 ±3.20	16.1 ±2.73	7.2 ±2.13	6.1 ±1.70	7.4 ±1.16	7.0 ±1.10	34.5 ±5.26	18.0 ±1.89	24.0 ±4.63	6.7 ±1.36	27.5 ±5.75	8.7 ±0.63

ml The change amounted to 8 7 $\pm$ 1 39 ml ($P < 0\ 001$) The simultaneously measured blood flow in the test hand decreased insignificantly from 17 0 $\pm$ 2 85 ml to 16 6 $\pm$ 2 45 ml (difference 0 4 $\pm$ 1 22 ml) - After the higher dose of hydralazine the mean increase of blood flow in the test hand was 7 6 $\pm$ 3 44 ml greater than that after the lower dose ($P < 0\ 05$)

B *Effect of Hydralazine on the Vasoconstrictor Action of the Cold Test in the Hand*

The cold test had little effect on the blood pressure In the three experiments in which this factor was studied, the systolic and diastolic blood pressure increased by 0–10 mm Hg The changes occurring before and after intra-arterial administration of hydralazine were of equal magnitude

1 Effect of the Higher Dose of Hydralazine

The effect of the cold test on the blood flow in the hands was studied in five experiments before and after administering the higher dose of hydralazine (table 1, figure 1) Before the infusion of hydralazine the cold test produced a quantitatively similar reduction of blood flow in the two hands After 10 seconds the mean decrease was 9 4 $\pm$ 3 19 ml in the test hand and 10 0 $\pm$ 2 69 ml in the control hand (difference 0 6 $\pm$ 0 64 ml), after 40 and 60 seconds, 9 3 $\pm$ 2 88 ml in the test hand and 9 2 $\pm$ 2 44 ml in the control hand (difference 0 1 $\pm$ 0 86 ml) After the administration of

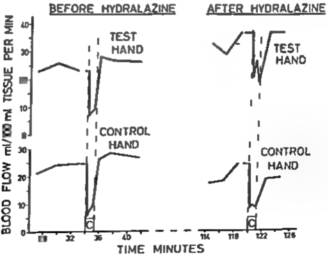


Fig 1 Effect of the cold test on blood flow in the hands before and after intra arterial administration of hydralazine Cold test performed at 34th and 120th minute Hydralazine (0 95 mg) given at 67th–72nd minute

Table I

Effect of the cold test on blood flow in the hands before and after intra arterial administration of hydralazine in the higher dose range Blood flow expressed as ml per 100 ml tissue per minute

Exp No	Hand volume ml		Intra arterial dose of hydralazine mg	Blood flow											
				Before hydralazine						After hydralazine					
				Basal		Cold test				Basal		Cold test			
						10 seconds		40 60 sec				10 seconds		40, 60 sec	
	Test hand	Contr hand		Test hand	Contr hand	Test hand	Contr hand	Test hand	Contr hand	Test hand	Contr hand	Test hand	Contr hand	Test hand	Contr hand
11	465	475	0 95	27 9	25 4	7 2	5 7	9 2	8 7	31 2	20 1	20 1	7 6	22 6	8 8
12	370	400	1 11	9 2	11 1	3 6	3 1	7 6	6 9	26 2	20 4	15 8	4 0	23 4	8 9
13	340	355	1 11	12 9	12 8	1 5	2 0	2 9	2 8	48 1	22 5	23 5	4 1	23 4	7 3
14	400	355	1 11	14 4	12 1	12 1	8 7	9 1	8 6	21 5	13 2	17 9	11 4	17 9	10 8
15	415	420	1 27	18 6	19 4	11 8	11 1	8 1	8 0	45 4	13 8	42 7	6 3	50 1	7 5
Mean value s. e m	398 ±21 1	401 ±22 4	1 11 ±0 051	16 6 ±3 20	16 1 ±2 73	1 2 ±2 13	6 1 ±1 70	7 4 ±1 16	7 0 ±1 10	34 5 ±5 26	18 0 ±1 89	24 0 ±4 85	6 7 ±1 36	27 5 ±5 75	8 7 ±0 63

hydralazine the cold test in two experiments produced a considerably smaller decrease of the blood flow in the test hand than in the control hand, but on average the absolute decrease was not significantly different in the hands After 10 seconds the mean decrease was 10 5 ± 3 92 ml in the test hand and 11 3 ± 3 02 ml in the control hand (difference 0 8 ± 2 23 ml), after 40 and 60 seconds, 7 0 ± 4 90 ml in the test hand and 9 3 ± 2 24 ml *in the control hand (difference 2 3 ± 3 66 ml)*

2 Effect of the Lower Dose of Hydralazine

In ten experiments the effect of a cold test on blood flow in the hand was investigated before and after infusion of the lower dose of hydralazine (table 2) The two cold tests evoked, on the average, roughly the same absolute decrease of blood flow in both hands After the administration of hydralazine the mean decrease 10 seconds after the application of the test was 9 9 ± 1 99 ml in the test hand and 10 1 ± 1 99 ml in the control hand (difference 0 2 ± 1 05 ml), the mean decrease after 40 and 60 seconds was 7 1 ± 1 20 ml in the test hand and 7 0 ± 1 05 ml in the control hand (difference 0 1 ± 0 90 ml)

C Effect of Hydralazine on the Vasoconstrictor Action of Noradrenaline in the Hand

Intravenous infusion of 20–24 μg noradrenaline per minute in three experiments elevated the systolic pressure by 10–25 mm Hg and the diastolic by 15–30 mm Hg The effect was of about the same magnitude before and after intra arterial administration of hydralazine

1 Effect of the Higher Dose of Hydralazine

a *Intravenous Noradrenaline* – In five experiments the effect of intravenous infusion of 12–24 μg noradrenaline per minute on blood flow in the hand was studied before and after intra arterial administration of the higher dose of hydralazine (table 3, figure 2) Before administration of hydralazine the decrease in blood flow produced by infusion of noradrenaline was the same in both hands, on the average, 6 9 ± 1 26 ml in the test hand and 6 7 ± 0 99 ml in the control hand (difference 0 2 ± 0 54 ml) After administration of hydralazine the infusion of noradrenaline reduced the blood flow in the test hand markedly less than in the control hand, the mean values being 2 2 ± 1 07 ml and 7 7 ± 1 25 ml respectively (difference 5 5 ± 1 62 ml, $P < 0.05$)

b *Intra arterial Noradrenaline* – In two experiments the effect on blood flow in the hand produced by an infusion of 0 27–0 36 μg nor-

Table [illegible]

Effect of the cold test on blood flow in the hands before and after intra arterial administration of hydralazine in the lower dose range. Blood flow expressed as ml per 100 ml tissue per minute

Exp No	Hand volume ml		Intra arterial dose of hydralazine mg	Blood flow											
				Before hydralazine						After hydralazine					
				Basal		Cold test				Basal		Cold test			
						10 seconds		40 60 sec				10 seconds		40 60 sec	
	Test hand	Contr hand		Test hand	Contr hand	Test hand	Contr hand	Test hand	Contr hand	Test hand	Contr hand	Test hand	Contr hand	Test hand	Contr hand
1	410	420	0.55	23.2	23.9	6.5	6.0	14.0	13.7	32.6	21.2	25.0	10.8	24.4	16.1
2	465	480	0.39	7.2	7.4	3.3	3.2	4.5	4.5	16.8	13.2	8.3	4.0	10.5	4.9
3	410	415	0.32	25.4	25.3	10.7	10.2	17.6	17.1	32.1	24.6	15.1	11.1	23.7	16.5
4	355	370	0.47	14.6	13.4	6.6	5.7	5.8	5.7	20.6	12.0	13.0	4.3	12.7	8.4
5	600	620	0.30	25.1	26.6	18.7	18.3	17.7	17.8	25.4	21.1	18.0	9.9	24.4	16.1
6	480	470	0.30	17.3	18.0	5.3	4.3	6.0	5.6	28.6	10.8	26.9	8.7	21.1	4.7
7	490	435	0.47	32.1	32.8	11.8	15.1	15.0	16.9	39.1	31.8	15.7	7.6	26.4	18.7
8	470	475	0.32	8.6	10.4	5.0	5.6	7.8	8.3	24.3	13.9	11.1	5.4	12.8	4.8
9	460	470	0.47	10.3	11.0	1.8	2.2	3.6	3.7	21.4	15.5	14.5	3.1	15.5	5.6
10	425	[illegible]	0.47	8.0	[illegible]	3.7	[illegible]	4.9	[illegible]	13.8	7.1	8.3	5.1	12.6	5.3

Table 3.

Effect of intravenous infusion of noradrenaline on blood flow in the hands before and after intra-arterial administration of hydralazine in the higher dose range Blood flow expressed as ml per 100 ml tissue per minute

Exp No	Hand volume ml		Intra arterial dose of hydralazine mg	Inravenous dose of noradrenaline μg/min	Blood flow							
					Before hydralazine				After hydralazine			
					Basal		Noradrenaline		Basal		Noradrenaline	
	Test hand	Contr hand			Test hand	Contr hand	Test hand	Contr hand	Test hand	Contr hand	Test hand	Contr hand
11	465	475	0 95	24	23 3	24 5	18 1	17 9	39 3	29 1	38 4	16 6
12	370	400	1 11	12	17 7	19 8	10 9	11 0	26 2	16 9	24 7	10 9
13	340	355	1 11	20	29 6	23 0	17 8	13 9	42 9	19 9	38 2	12 2
14	400	355	1 11	20	16 0	12 5	10 3	8 4	19 6	11 0	20 4	5 6
15	415	420	1 27	20	11 8	12 5	6 7	7 4	43 9	15 2	39 4	8 0
Mean value s e m	398 ±21 1	401 ±22 4	1 11 ±0 051	19 ±2 0	19 7 ±3 09	18 4 ±2 55	12 8 ±2 24	11 7 ±1 91	34 4 ±1 54	18 4 ±3 03	32 2 ±4 01	10 7 ±1 88

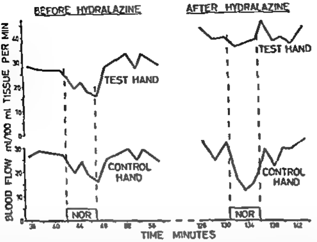
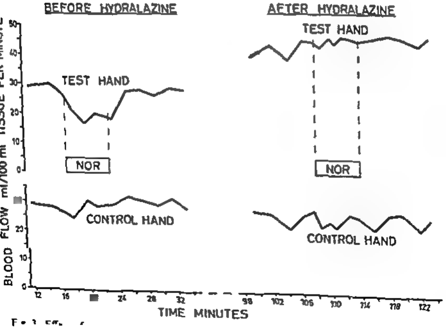


Fig 2 Effect of intravenous infusion of noradrenaline on blood flow in the hands before and after intra arterial administration of hydralazine Noradrenaline (24 µg per minute) given at 42nd–47th and 131st–136th minute Hydralazine (0 95 mg) given at 67th–72nd minute



adrenaline per minute into one brachial artery was studied before and after administering the higher dose of hydralazine The results obtained in one experiment are shown in fig 3 Before administration of hydralazine the infusion of noradrenaline caused in both experiments a conspicuous decrease of blood flow in the test hand as compared with the simultaneously recorded flow in the control hand After administration of hydralazine the noradrenaline infusion caused practically no decrease of blood flow in the test hand

2 Effect of the Lower Dose of Hydralazine

a *Intravenous Noradrenaline* – In six experiments a study was made of the effects of intravenous infusion of 12–20 μg noradrenaline per minute before and after intra-arterial administration of the lower dose of hydralazine (table 4) Before the administration of hydralazine the infusion of noradrenaline produced on an average a decrease in the blood flow of 7 0 ± 1 67 ml in the test hand and of 7 7 ± 1 87 ml in the control hand (difference 0 7 ± 0 37 ml) After the administration of hydralazine the infusion of noradrenaline produced in every experiment a distinct decrease in the blood flow in the test hand On an average the absolute decrease was of about the same magnitude in both hands, in the test hand 8 3 ± 2 57 ml and in the control hand 7 7 ± 1 58 ml (difference 0 6 ± 1 34 ml) The results thus differed from those obtained in experiments in which a higher dose of hydralazine was given

b *Intra arterial Noradrenaline* In five experiments an intra arterial infusion of 0 15–0 42 μg noradrenaline per minute was given before and after administering the lower dose of hydralazine into the same brachial artery (table 5) Before the administration of hydralazine the infusion of noradrenaline reduced the blood flow in the test hand in all experiments (mean decrease 5 7 ± 1 32 ml, P < 0 05), but variable changes occurred in the control hand (on the average, a decrease of 0 4 ± 0 69 ml) If, as believed, the changes in the control hand resulted from spontaneous variations in the vasoconstrictor fibre discharge similar changes must likewise have occurred in the test hand and modified the vasoconstrictor effect of the infusion of noradrenaline By assuming that these spontaneous changes were of the same absolute magnitude in both hands in each experiment, the "real" effect of the infused noradrenaline could be calculated An average decrease of 5 3 ± 1 03 ml (P < 0 05) was found After hydralazine, noradrenaline also reduced blood flow in the test hand in every experiment If the "real" effect of noradrenaline was calculated in the same way as above, the average decrease was 5 9 ± 0 67 ml (P < 0 01) Thus noradrenaline produced about the same decrease of blood

Table 4

Effect of intravenous infusion of noradrenaline on blood flow in the hands before and after intra arterial administration of hydralazine in the lower dose range Blood flow expressed as ml per 100 ml tissue per minute

Exp No	Hand volume ml		Intra arterial dose of hydralazine mg	Intravenous dose of noradrenaline µg/min	Blood flow							
					Before hydralazine				After hydralazine			
					Basal		Noradrenaline		Basal		Noradrenaline	
	Test hand	Contr hand			Test hand	Contr hand	Test hand	Contr hand	Test hand	Contr hand	Test hand	Contr hand
1	410	420	0 55	20	19 3	20 2	9 0	10 0	37 6	23 9	19 7	9 4
2	465	480	0 39	12	6 3	5 5	3 9	3 3	19 1	13 3	16 0	6 7
3	430	415	0 32	12	19 1	21 3	11 9	12 1	26 1	20 6	20 5	13 7
4	355	370	0 47	20	11 8	11 6	9 7	9 3	16 6	8 6	13 7	5 8
5	600	620	0 30	20	27 7	28 6	15 5	14 9	20 8	14 9	14 9	8 4
6	480	470	0 30	20	15 1	14 5	7 1	6 1	31 9	14 7	17 5	5 8
Mean value s e m	460 ±33 1	463 ±35 5	0 39 ±0 042	17 ±1 7	16 5 ±2 99	17 0 ±3 32	9 5 ±2 64	9 3 ±2 86	25 4 ±3 32	16 0 ±4 95	17 1 ±1 10	8 3 ±1 51

Table 5

Effect of intra arterial infusion of noradrenaline on blood flow in the hands before and after intra arterial administration of hydralazine in the lower dose range Blood flow expressed as ml per 100 ml tissue per minute

Exp No	Hand volume ml		Intra arterial dose of hydralazine mg	Intraarterial dose of noradrenaline µg/m1	Blood flow							
					Before hydralazine				After hydralazine			
					Basal		Noradre naline	Basal	Basal		Noradre naline	Basal
	Test hand	Contr hand			Test hand	Contr hand	Test hand	Contr hand	Test hand	Contr hand	Test hand	Contr hand
5	600	620	0 30	0 32	21 8	23 1	14 9	22 6	28 0	21 1	21 3	20 3
6	480	470	0 30	0 15	13 5	13 0	9 3	13 6	27 1	10 6	19 7	9 9
7	490	435	0 47	0 42	33 4	35 6	23 4	34 2	36 6	34 0	31 8	33 2
8	470	475	0 32	0 18	12 1	14 1	6 9	11 7	18 6	7 6	15 1	9 2
10	425	400	0 47	0 36	7 3	7 1	5 1	8 6	19 0	7 6	12 5	9 0
Mean value s e m	493 ±29 0	480 ±37 5	0 37 ±0 040	0 29 ±0 052	17 6 ±4 58	18 6 ±4 97	11 9 ±3 31	18 1 ±4 64	25 9 ±3 33	16 2 ±5 10	20 1 ±3 33	16 3 ±4 72

flow in the test hand after as before hydralazine was given (mean difference 0 8 ± 1 51 ml), and this result also differed from that obtained when a higher dose of hydralazine was given

Discussion

A great part of the blood vessels in the hand are engaged in temperature regulation, and their resistance is to a large extent determined by the activity of the vasoconstrictor nerves (*cf* review by HERTZMAN 1959) According to ARNOTT *et al* (1948), GASKELL (1956) and RODDIE *et al* (1957 a, b) neurogenic vasoconstrictor tone in the fingers and the hand can be eliminated completely by indirect heating, since this procedure reduces vascular resistance in the hand to the same degree as does nerve block However, the resistance vessels in the hand are evidently not completely relaxed by indirect heating, since RODDIE & SHEPHARD (1956) found that they could be further dilated by local heating This observation indicates that the hand contains vessels with an inherent tone not maintained by vasoconstrictor nerve activity

In our experiments the higher dose of hydralazine appeared to exert a pronounced antagonism to the vasoconstrictor effect of noradrenaline in the hand whereas the effect of the cold test was antagonized to a smaller degree This may indicate that hydralazine exerted a weaker antagonism to neurogenic vasoconstrictor stimuli than to plasma noradrenaline, but this question needs further study The lower dose of hydralazine evoked a distinct decrease in vascular resistance, but the vasoconstrictor effects of noradrenaline and of the cold test were probably only slightly antagonized This indicates that the vasodilator action of hydralazine in the hand is primarily due to a "direct" effect on vascular smooth muscle At lower dosage the drug reduces vascular resistance without severely interfering with vasoconstrictor fibre control of resistance At higher dosage it may exert a pronounced antagonism to the assumed adrenergic transmitter noradrenaline

In order to test this interpretation, we have also investigated if the effects of hydralazine could be distinguished from the effects of peripherally acting agents that produce vasodilatation by release of vasoconstrictor tone (unpublished) Two such drugs were studied the adrenergic blocking agent phenoxybenzamine and guanethidine, which according to MAXWELL *et al* (1960) and others inhibits the release of the adrenergic transmitter from the nerve endings These drugs were infused into a brachial artery and evoked a long lasting decrease in vascular resistance of the hand They were given at a dose producing about the same increase in blood flow of the test hand as the lower dose of hydralazine, and their interac-

tion with vasoconstrictor stimuli was studied in the same way as in the experiments with hydralazine After phenoxybenzamine the vasoconstrictor effects of the cold test and noradrenaline in the test hand were markedly reduced After guanethidine the effect of the cold test in the test hand was reduced but the effect of noradrenaline was either not affected or even possibly somewhat potentiated These results differ from those obtained with hydralazine and therefore lend support to the interpretation made above for the mechanism of the vasodilatory action of hydralazine

It has earlier been suggested by STUNKARD *et al* (1954) that a peripheral action of this type is of predominant importance for the hypotensive effect of hydralazine in man They found that an intravenous injection of 8–22 mg hydralazine increased the blood flow in extremities devoid of sympathetic innervation and that it evoked a hypotensive effect, but did not quantitatively change the absolute increase in blood pressure evoked by a cold test or intravenously infused noradrenaline On the other hand it has been shown that higher doses of intravenously administered hydralazine sometimes decrease the hypertensive effect of these stimuli in man (FREIS *et al* 1950, 1951, GRIMSON *et al* 1950) Also in animal experiments the drug has sometimes been found to inhibit the hypertensive effect of noradrenaline completely and convert the pressor effect of adrenaline to a depressor effect (e g MOYER *et al* 1951) The vascular effects of locally administered hydralazine in the hand thus seem to be representative of the effects produced by intravenous administration of the drug on the entire peripheral vascular bed

In man the effects of oral or parenteral hydralazine on the vascular resistance in various organs support the view that the vasodilation is predominantly due to a "direct" action on the vascular smooth muscle The drug generally evokes a considerable increase of renal blood flow (REUBI 1950, WILKINSON *et al* 1952, KAPLAN & ASSALI 1953, MOYER 1953, WERKÖ *et al* 1953, CROSLEY *et al* 1954, VAN DER KOLK *et al* 1954, STEIN & HECHT 1955, GJORUP & HILDEN 1956, JUDSON & HOLLANDER 1956) This effect is probably not due to a decrease in neurogenic vasoconstrictor tone since this factor is considered to be of no importance for the renal vascular resistance under basal conditions in man (review by SMITH 1951) The effect of hydralazine on cerebral blood flow in man has been studied in several investigations In one study the blood flow was not changed by the drug (HAFKENSCHIEHL & FRIEDLAND 1953) but in three others a distinct increase was found (CRUMPTON *et al* 1953, MCCALL 1953, KLEH & FAZEKAS 1954) Vasoconstrictor nerve activity is considered to be of little importance for the vascular resistance in this region, hypotensive drugs that primarily inhibit the neurogenic vascular tone do not

increase cerebral blood flow, whereas "directly" acting spasmolytic drugs, such as nitrites and papaverin, may increase the flow (*cf* review by SOKOLOFF 1959) Hydralazine has been shown also to increase the blood flow and decrease the arterio-venous oxygen difference in the coronary circulation (ROWE *et al* 1955), where the vascular resistance is believed to be but little influenced by vasoconstrictor nerve activity

Summary

The interaction of hydralazine with the vasoconstrictor effects of noradrenaline and a cold test was studied in the hand When infused into a brachial artery, hydralazine produced in the hand vasodilatation of gradual onset and long duration When administered in a higher dose of 0 95–1 27 mg the drug appreciably antagonized the vasoconstrictor action of intravenously or intra arterially infused noradrenaline, but antagonized the effect of the cold test to a less extent When administered in a lower dose of 0 30–0 55 mg, hydralazine elicited significant vasodilatation, but did not markedly antagonize the vasoconstrictor stimuli The results are in accordance with previous investigations, which have shown that 1 hydralazine has a peripheral vasodilator effect due to a "direct" action on vascular smooth muscle, 2 hydralazine's peripheral site of attack is of paramount significance for its hypotensive action

Acknowledgement

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In man, the effects of oral or parenteral hydralazine on the vascular resistance in various organs support the view that the vasodilation is predominantly due to a ' direct" action on the vascular smooth muscle The drug generally evokes a considerable increase of renal blood flow (REUBI 1950, WILKINSON *et al* 1952, KAPLAN & ASSALI 1953, MOYER 1953, WERKÖ *et al* 1953, CROSLEY *et al* 1954, VAN DER KOLK *et al* 1954 STEIN & HECHT 1955, GJORUP & HILDEN 1956, JUDSON & HOLLANDER 1956) This effect is probably not due to a decrease in neurogenic vasoconstrictor tone, since this factor is considered to be of no importance for the renal vascular resistance under basal conditions in man (review by SMITH 1951) The effect of hydralazine on cerebral blood flow in man has been studied in several investigations In one study the blood flow was not changed by the drug (HAFKENSCHIEHL & FRIEDLAND 1953) but in three others a distinct increase was found (CRUMPTON *et al* 1953, MCCALL 1953, KLEH & FAZEKAS 1954) Vasoconstrictor nerve activity is considered to be of little importance for the vascular resistance in this region, hypotensive drugs that primarily inhibit the neurogenic vascular tone do not

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The Combined Effect of Adrenaline Desoxicortone and Sodium Chloride on Blood Pressure in the Rat

By

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In our earlier study (AHO *et al* 1961) we reported that continuous prolonged treatment of rats with adrenaline in oil increased the effect on blood pressure of simultaneous NaCl treatment Prolonged injections of adrenaline over several months may slightly increase the blood pressure of some experimental animals (ENGER 1940, SCHROEDER & NEUMAN 1942, HEIM 1952, AHO *et al* 1961)

The acute blood pressure response to either injected or infused adrenaline or noradrenaline is more pronounced in man (RAAB *et al* 1950, RAAB 1953) and to noradrenaline in the dog (VANATTA & COTTLE 1955) after pretreatment with both desoxicortone (DOC) and DOC + NaCl Adrenalectomy decreases the pressor effects of noradrenaline (FRITZ & LEVINE 1951) Intact and bilaterally nephrectomized rats show about equally great increases in blood pressure after single injections of adrenaline and noradrenaline, whereas the acute rise in blood pressure of unilaterally nephrectomized rats after pretreatment with DOC + NaCl generally is higher (GROSS & LICHTLEN 1958)

It is wellknown that hypertension can be caused in rats by combined treatment with DOC and NaCl, DOC alone has generally not been effective, but combined with a diet containing NaCl it can cause experimental hypertension (KNOWLTON *et al* 1946, SELYE *et al* 1949, BRANDT *et al* 1951, GROSS *et al* 1956, ERÄNKÖ *et al* 1955) Different kinds of animals have shown different responses As test animals rats have generally been used

In intact rats DOC treatment slightly increases the blood pressure, and simultaneous NaCl intake intensifies this (FRIEDMAN *et al* 1948) In most investigations of hypertension caused by DOC + NaCl unilateral ~hrectomy has been used as a sensitizing factor (SELYE 1943, 1950, ~ss *et al* 1956) In nephrectomized rats this hypertension is regularly

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The Combined Effect of Adrenaline Desoxicortone and Sodium Chloride on Blood Pressure in the Rat

By

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In our earlier study (AHO *et al* 1961) we reported that continuous prolonged treatment of rats with adrenaline in oil increased the effect on blood pressure of simultaneous NaCl treatment Prolonged injections of adrenaline over several months may slightly increase the blood pressure of some experimental animals (ENGER 1940, SCHROEDER & NEUMAN 1942, HEIM 1952, AHO *et al* 1961)

The acute blood pressure response to either injected or infused adrenaline or noradrenaline is more pronounced in man (RAAB *et al* 1950, RAAB 1953) and to noradrenaline in the dog (VANATTA & COTTLE 1955) after pretreatment with both desoxicortone (DOC) and DOC + NaCl Adrenalectomy decreases the pressor effects of noradrenaline (FRITZ & LEVINE 1951) Intact and bilaterally nephrectomized rats show about equally great increases in blood pressure after single injections of adrenaline and noradrenaline, whereas the acute rise in blood pressure of unilaterally nephrectomized rats after pretreatment with DOC + NaCl generally is higher (GROSS & LICHTLEN 1958)

It is wellknown that hypertension can be caused in rats by combined treatment with DOC and NaCl, DOC alone has generally not been effective but combined with a diet containing NaCl it can cause experimental hypertension (KNOWLTON *et al* 1946, SELYE *et al* 1949, BRANDT *et al* 1951, GROSS *et al* 1956, ERANKO *et al* 1955) Different kinds of animals have shown different responses As test animals rats have generally been used

In intact rats DOC treatment slightly increases the blood pressure and simultaneous NaCl intake intensifies this (FRIEDMAN *et al* 1948) In most investigations of hypertension caused by DOC + NaCl unilateral nephrectomy has been used as a sensitizing factor (SELYE 1943, 1950, GROSS *et al* 1956) In nephrectomized rats this hypertension is regularly

accompanied by increased heartweight, kidneyweight and bloodvolume (PICKERING 1955) The decrease in renal tissue seems to be of great importance for the appearance of experimental hypertension (BRAUN MENENDEZ 1958)

Certain emotional factors, the importance of which in essential hyper tension is generally recognized, may also increase the secretion of adrena line (v EULER & LUNDBERG 1954, 1959, ELMADJIAN *et al* 1956, 1958 PEKKARINEN *et al* 1961)

As the studies mentioned above generally support the opinion that DOC strengthens the acute blood pressure effect of adrenaline, we have thought it useful to study whether prolonged treatment with adrenaline strengthens the effects of either DOC or of DOC and NaCl on the blood pressure in intact rats

Material and Methods

The test animals used were albino rats with an average weight of 230 g In the adrenaline + DOC group there were 11, in the DOC group 11 in the DOC + NaCl group 3 and in the adrenaline + DOC + NaCl group 9 rats The adrenaline base diluted in oil to a concentration 1 200 (Adrenaline Retard Leo in an oil solution containing ascorbic acid 0 015 white beeswax 0 02 anhydrous lanoline 0 04 and arachis oil in 1 ml) was daily injected subcutaneously into the dorsal region near the median line the dose was 1 mg/rat As the DOC preparation 25 mg of desoxycorti costerone oenanthate (Primocort Depot ® Leiras Schering) per rat were injected subcutaneously every two weeks 1–2% NaCl was given as drinking water Bloodless measurements of the blood pressure were carried out at 5 14 days intervals on the hind leg by means of a photoelectric tensometer (KERSTEN *et al* 1947) (Metro Indu stries) without anaesthesia The blood pressure was measured in the following day after injections Before the blood pressure measurement the rats were kept for 5 10 minutes in animal holders To prevent infection syringes and needles were sterilized in boiling water Most of the animals survived the treatment but 5 of them died

Statistical Treatment

In the mathematical analysis the values of blood pressure in the adrenaline + DOC group were compared with those in the DOC group and the values in the adrenaline + DOC + NaCl group with the corresponding values in the DOC + NaCl group Student s p test was used and the difference was considered significant if $p \geqq 0\ 05$ and highly significant if $p \geqq 0\ 01$

Results

1 *The DOC group* (11 rats) Fig 1 At the beginning of the test the mean blood pressure was $107 \pm 4\ 0$ mm Hg During the first month it rose to $118 \pm 3\ 5$ mm Hg and during the second to $136 \pm 5\ 5$ mm Hg The mean maximum rise in blood pressure was 29 mm Hg During the second month of treatment 3 values were above 160 mm Hg During the

third month of treatment the mean blood pressure showed a tendency to fall to 125 ± 4 0 mm Hg No individual blood pressure value was above 165 mm Hg during the third month of treatment Blood pressure values of 150 mm Hg or more were found in 8 (19%) measurements and 130 mm Hg or more in 25 (60%) during the third month of treatment

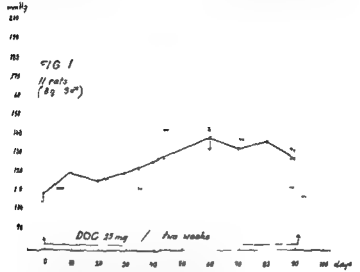

Fig 1 The effect of prolonged treatment (3 months) with DOC oenanthate (25 mg s c every 2 week) upon the blood pressure of rats

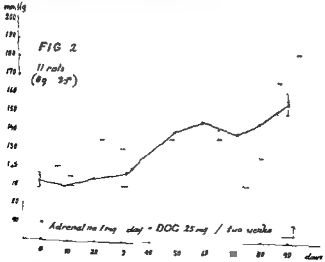

Fig 2 The effect of combined and prolonged treatment (3 months) with adrenaline in oil (s c 1 mg each day) and DOC oenanthate (25 mg s c every 2 week) upon the blood pressure of rats

2 *The adrenaline + DOC group* (11 rats) Fig 2 The mean initial pressure was 112 ± 4 0 mm Hg The pressure remained at that level during the first month, increased during the second month to 144 ± 5 0 mm Hg, after the third month it was 154 ± 5 5 mm Hg During the test the mean maximum rise in blood pressure was 42 mm Hg The increase in blood pressure continued during the whole period of treatment The highest individual values for blood pressure were found during the third month of treatment, 180 mm Hg or more in 4 out of 31 measurements (13%), 150 mm Hg or more were found in 12 measurements (39%) and the blood pressure was above 130 mm Hg in 19 measurements (61%)

3 *The DOC + NaCl group* (3 rats) Fig 3 At the beginning the mean blood pressure was 116 ± 5 0 mm Hg, it rose during the first month to 127 ± 4 5 mm Hg and during the second to its maximum, 135 mm Hg after which it again fell, reaching on average 130 ± 6 0 mm Hg at the end of the third month The mean maximum rise in blood pressure was only 19 mm Hg, but the rise did not continue uniformly during the whole test period The individual blood pressure values were always under 150 mm Hg

4 *The adrenaline + DOC + NaCl group* (9 rats) Fig 4 The blood pressure was at the beginning 119 ± 4 0 mm Hg, after one month 130 ± 5 5 and after two months 148 ± 4 0 mm Hg The rise continued during the third month up to 157 ± 4 0 mm Hg, after this, when the treatment had been stopped, there was a fall in the blood pressure The mean maximum rise in blood pressure was thus 38 mm Hg, it continued for the whole test period The blood pressure was once more than 180 mm Hg, in 11 times (41%) above 150 mm Hg and 21 times (84%) above 130 mm Hg

We thus observed that adrenaline during a chronic treatment only potentiates to a limited extent the blood pressure effect of either DOC or DOC + NaCl In the adrenaline + DOC group the mean blood pressure (154 ± 5 5 mm Hg) is only highly significantly higher ($p < 0\ 01$) than in the DOC group (125 ± 4 0 mm Hg) by the end of the test In DOC + NaCl group the number of animals was too small for comparison with the other groups

DOC treatment alone increased the blood pressure to 130–136 mm Hg The adrenaline + DOC + NaCl and the adrenaline + DOC groups showed a tendency toward continuous increase in blood pressure but scarcely any difference could be observed between the two groups The NaCl in these tests did not show any further potentiating effect on the adrenaline and DOC group or the DOC group The DOC + adrenaline treatment increased the blood pressure to a somewhat higher level than

the adrenaline treatment did alone in our earlier studies (AHO *et al* 1961) Similarly the DOC adrenaline and NaCl treatment increased the blood pressure to a somewhat higher level than only the adrenaline and NaCl treatment combined (AHO *et al* 1961) However, the difference in blood pressure between these two groups was small

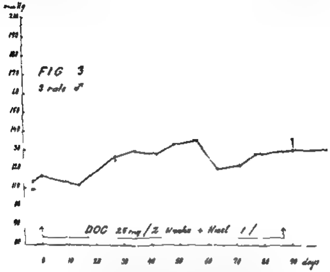

Fig 3 The effect of combined treatment with DOC oenanthate (25 mg s c every second week) and 1% NaCl as drinking water upon the blood pressure of rats

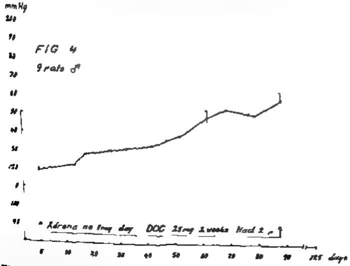

Fig 4 The effect of combined and prolonged treatment with adrenaline in oil (1 mg s c each day) and DOC oenanthate (25 mg s c every second week) and 2% NaCl in drinking water upon the blood pressure of rats

2 *The adrenaline + DOC group* (11 rats) Fig 2 The mean initial pressure was 112 ± 4 0 mm Hg The pressure remained at that level during the first month, increased during the second month to 144 ± 5 0 mm Hg, after the third month it was 154 ± 5 5 mm Hg During the test the mean maximum rise in blood pressure was 42 mm Hg The increase in blood pressure continued during the whole period of treatment The highest individual values for blood pressure were found during the third month of treatment, 180 mm Hg or more in 4 out of 31 measurements (13%), 150 mm Hg or more were found in 12 measurements (39%) and the blood pressure was above 130 mm Hg in 19 measurements (61%)

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other additional sensitizing factors, for example, unilateral nephrectomy, were generally needed (SELYE 1943, 1949, ERANKO & NIEMI 1955; GROSS *et al* 1956) A high NaCl intake and large doses of corticosteroids along with nephrectomy caused elevation of the blood pressure, nephrosclerotic changes and cardiac hypertrophy (SELYE *et al* 1943)

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In earlier studies in male rats, with 10% NaCl in the drinking water, with two subcutaneous implants of 40 mg pellets of DOC and with unilateral nephrectomy, there were no clearly increased vascular responses to adrenaline renin or angiotonin (MASSON *et al* 1950)

It is well known, that adrenaline and noradrenaline greatly increase renal vasoconstriction and can cause renal ischemia (GOLDZIEHER 1946, TRUETA *et al* 1947, CERLETTI *et al* 1949, HARTMAN & BROWNELL 1949) They may enhance the tendency to sodium retention and increase the blood pressure Emotional factors can also diminish the renal blood flow (WOLFF 1953)

Giving DOC alone increases the blood pressure only a little, thus after 6 weeks it rises to 117 ± 18 8 mm Hg, the control group being at 108 ± 14 8 mm Hg (FRIEDMAN *et al* 1948) No effect on blood pressure was noted with DOC alone (5 mg Percorten subcutaneously in three months) (GROSS 1950) In our own studies slight increase in blood pressure was observed during DOC treatment The blood pressure in rats had risen to 158 mm Hg in 10 weeks after a subcutaneously implanted 20 mg DOC pellet (GREEN *et al* 1948)

The adrenocortical hormones as well as the sympathetic nervous system are considered to be important for the maintenance of normal blood pressure These effects seem to depend on the power of the mineral hormones to accumulate sodium intracellularly, whereby the sodium gradient aeron the cell membrane is decreased and the vascular tone and contractile responsiveness are accordingly raised (RAAB 1959) The sodium ion gradient aeron the membrane plays a fundamental role in blood pressure regulation (FRIEDMAN *et al* 1957) The catecholamines decrease the sodium and potassium gradients and thus potentiate the mineralocorticoid action on the vascular cells and the increase in blood pressure (RAAB 1959)

Discussion

Since the labile neurogenic increase in blood pressure plays an important part in the development of essential hypertension, we have studied the role of prolonged injections of adrenaline on the development of experimental hypertension in rats (AHO *et al* 1961) In our experiments the increase in blood pressure was caused by adrenaline, DOC and NaCl treatment Injection pain only caused a small increase in the mean blood pressure in the rat (LINNA *et al* 1962) In some individuals we obtained the highest values of blood pressure in both the groups treated additionally by prolonged injections of adrenaline Similarly, individual values of blood pressure above 150 mm Hg were observed more often in the two groups treated additionally by adrenaline (39 and 41 %), whereas in the group receiving DOC alone it was above 150 mm Hg in only 19% of the animals and in the DOC + NaCl group it was always under 150 mm Hg The mean increase in blood pressure, although clear during adrenaline + DOC + NaCl treatment, was not greater than during adrenaline + DOC treatment In an earlier study (RAAB *et al* 1950) pretreatment with DOC enhanced the acute rise in blood pressure with adrenaline and noradrenaline in man, but addition of NaCl did not increase it further In acute adrenaline tests clear sensitizing effects of DOC were observed (RAAB 1953, VANATTA & COTTLE 1955) In hypertension there is an increased vascular reaction to catecholamines (CROUT 1959)

In our experiments the rise in blood pressure of rats during the adrenaline + DOC and adrenaline + DOC + NaCl treatments was slight except in a few individuals, despite the large doses of DOC, if the values 160–200 mm Hg are considered to be clearly elevated blood pressure values in experimental hypertension of rats

As in earlier studies with adrenaline (HEIM 1952) and adrenaline + NaCl (AHO *et al* 1961), there were observed rather large fluctuations in the individual blood pressure curves during both the adrenaline + DOC and the adrenaline + DOC + NaCl treatment

It is important to remember that DOC is not a natural hormone produced to any greater extent, so that its effects on the blood pressure does not correspond with the organism's own normal mechanism But a large excess of aldosterone along with NaCl treatment also increases blood pressure in the rat (GROSS *et al* 1957) During chronic adrenaline, DOC and NaCl treatment some "buffer" mechanisms were able partly to compensate for the increase of the blood pressure in our experiments Probably the normal organism has buffer mechanisms to compensate the effects of such large amounts of adrenaline, DOC and NaCl

In earlier studies of experimental hypertension with DOC and NaCl,

other additional sensitizing factors, for example, unilateral nephrectomy, were generally needed (SELYE 1943, 1949, ERANKÖ & NIEMI 1955, GROSS *et al* 1956) A high NaCl intake and large doses of corticosteroids along with nephrectomy caused elevation of the blood pressure, nephrosclerotic changes and cardiac hypertrophy (SELYE *et al* 1943)

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Summary

The effect of adrenaline in oil upon the blood pressure of intact rats was studied during 3 months prolonged treatment, by administering it simultaneously with desoxicortone (DOC) and DOC + NaCl. The adrenaline dose was 1 mg/day/rat and that of DOC was 25 mg/two weeks/rat, subcutaneously; 2 per cent NaCl was given in the drinking water. The blood pressure was measured without bloodshed by a photoelectric tensometer and without anaesthesia. In the adrenaline + DOC group the mean blood pressure (154 $\pm$ 5.5 mm Hg) at the end of the test was significantly higher than in the DOC group (125 mm Hg). During the period between 50–80 days, the mean blood pressure was also significantly higher in the adrenaline + DOC + NaCl group than in the DOC + NaCl group, but the difference was not so significant at the end of the test. After 3 months treatment the mean blood pressure was 157 mm Hg in the adrenaline + DOC + NaCl group and 130 mm Hg in the DOC + NaCl group

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At the end of the paper, all references should be given with the surnames *in the same order as that by the original authors* The initials (*or given names*) of the first author of a paper is to follow his surnames, those of the other authors are to precede them *The full title is next given in the original language*, and then the title of the journal (abbreviated in accordance with international custom and underlined in the manuscript) year of publication, number of volume (double underlined) and the numbers of the first and *last* pages

Examples

Vogt Marthe The secretion of the denervated adrenal medulla of the cat *Brit J Pharmacol* 1952 7 325 330

Funke A B H R F Rekker M J E Ernsting H M Tersteege & W Th Nauta Untersuchungen über Spasmolytica V Benzilsäureester *Arzneimittelforsch* 1959 9 573–576

REFERENCE

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Acta pharmacol et toxicol 1962, 19, 191–198

Department of Medical Chemistry, University of Turku
Turku 3, Finland

Effect of Aminoacetonitrile, Iproniazid and Semicarbazide on the Tensile Strength of Experimental Granulation Tissue

By

J. Viljanto, H. Isomäki and E. Kulonen

(Received April 9 1962)

Aminoacetonitrile and β-aminopropionitrile decrease the tensile strength of skin wounds (Kalliomäki, Yli-Pohja & Kulonen 1957) and increase the fragility of embryonal chick tissues (Levene & Gross 1959), which are among the symptoms of experimental lathyrism It is further established that iproniazid and semicarbazide aggravate the lathyrism (Roy, Lipton, Strong & Bird 1959, Juva, Mikkonen, Tuominen & Kulonen 1959) and even cause its symptoms alone (Dasler 1958, Neuman, Maxwell & McCoy 1959)

The purpose of the experiments reported here was to explore the effect of the substances mentioned on the tensile strength of granulation tissue by a method permitting simultaneous chemical analysis Levene (1961) studied recently the effect of numerous substances on the solubility of collagen and on the fragility of chick embryos, it will be seen below that our results agree well with his

Experimental

Animals Altogether 421 rats of Wistar origin (weight 99 ± 20 g in the beginning of the experimental periods) were used After implantation they were kept in steel wire cages 2 in each and allowed to eat the standard food *ad libitum* They were weighed weekly but the sexes were not recorded

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used but comparisons were made only between granulomata produced by the same materials [illegible]

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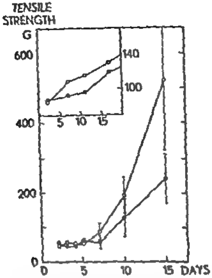


Fig 2 Effect of administering semicarbazide (● 25 mg/100 g daily, ○ control) on the tensile strength of granulation tissue Inserted is the average weight (in g) of the animals

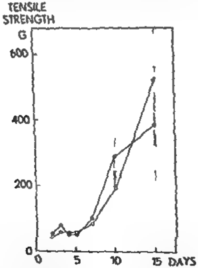


Fig 3 Effect of administering iproniazid (● 5 mg/100 g daily, ○ control) on the tensile strength of granulation tissue

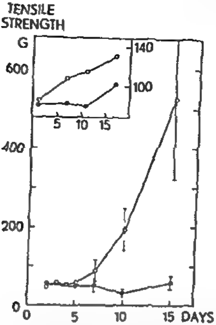


Fig 1 Effect of administering aminoacetonitrile (● 25 mg/100 g daily ○ control) on the tensile strength of granulation tissue Inserted is the average weight (in g) of the animals

about 25, 50 or 100 mg/100 g body weight Semicarbazide hydrochloride (E Merck, Darmstadt) was also dissolved in distilled water and neutralized with sodium hydroxide The solution was mixed with the food, and the rats received daily about 25 mg/100 g body weight Iproniazid (1-*iso*nicotinyl-2-*iso*propylhydrazide) was used as "*Marsilid*" tablets (*F Hoffman-La Roche & Co A G, Basel*), which were ground in a mortar, suspended in distilled water, shaken for at least 3 hours and neutralized with sodium hydroxide The daily dosage was 2, 5 or 10 mg/100 g body weight

Chemical analyses Homogenized granulomata were combined to give samples containing 3–8 (in the average 5 3) sponge implants The nitrogen was determined by micro-Kjeldahl combustion and subsequent distillation the hydroxyproline by the method of Neuman & Logan (1950)

Results

Aminoacetonitrile The dose of 100 mg/100 g/day was too toxic, and about half of the animals died from it shortly after implantation At a dose of 25 mg/100 g/day none of the animals died, but even then the gain in body weight was appreciably reduced Statistical treatment showed that at this dosage the decrease in tensile strength was significant (on 7th day $P < 0\,05$, on 10th day $P < 0\,001$), as indicated in fig 1 (see also fig 5 for hydroxyproline and fig 6 for total nitrogen)

Semicarbazide. A clear decrease (fig 2) was observed both in weight gain and in tensile strength ($P < 0\,01$ on 10th day)

Iproniazid The preliminary experiment (fig 3) indicated that iproniazid may enhance the tensile strength on the 3rd and 10th days ($P < 0\,01$)

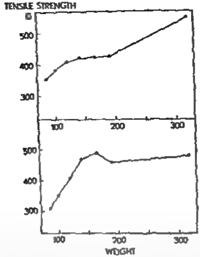


Fig 4 Average tensile strength of granulation tissue (below) and of skin wound (top, 1 cm broad strips) in untreated animals of different weights (12 days after operation)

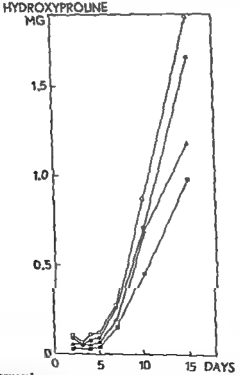


Fig 5 Total hydroxyproline per sponge implant at different times after implantation (○ control, ● 5 mg/100 g iproniazid daily, ▲ semicarbazide 25 mg/100 g daily, ■ aminoacetonitrile 50 mg/100 g daily)

Table 1

The effect of various treatments with iproniazid on tensile strength in experimental granulomata in rats

Iproniazid mg/day/rat	No of rats	Beginning of treatment	Average weight in start	Weight gain g/day	Tensile strength g/sponge implant	
					3rd post operational day	12th post operational day
Control	27	–	116±16	2 26	44±12	465±95
2	21	7 days before operation	112±15	1 53	44±12	445±66
5	21	14 days before operation	111±12	1 42	35±12	429±70
5	37	at operation	116±16	0 50	31± 3[1])	459±93
5	21	7 days before operation	113±12	0 42	38± 8	439±67
10	21	7 days before operation	115± 9	0 21	32± 6[2])	414±97

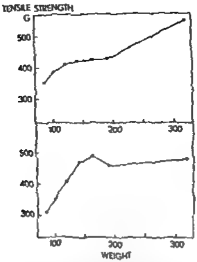
The differences (*vs* control) were statistically non significant, except in groups marked [1]) ($P < 0\ 02$) and [2]) ($P < 0\ 01$)

This finding was later proved misleading, but it prompted a check with a series of 148 animals (Table I) It can be concluded that the effect on the tensile strength is always negative It is not clear whether the effect of iproniazid is due to a non-specifically impaired general condition

Effect of the weight of the rat Twenty-eight rats were divided into three groups according to the weight 101 ± 10 g, 167 ± 20 g and 318 ± 45 g (ages 45, 70 and 180 days, respectively) After implantation of the "Vi-sella"-sponges, the tensile strength of implant tissue and skin wounds was measured After three days there were no clear differences in granulomata, but the skin wounds were less strong ($P < 0\ 01$) in the group of smallest animals At the 12th day the tensile strength of the skin wounds seemed to be almost proportional to the weight of the animals, but in the granulomata the differences were not statistically significant

To get further evidence on this point, all the results from untreated animals were divided into seven groups according to the weight of the animals The averages of the tensile strength were plotted against the weight (fig 4) The strength of skin scars seems to increase continuously, presumably because of the thicker skin in older animals The strength of the granulation tissue seems to depend on the weight of the animal up to a certain limit (about 140 g)

Chemical analyses It is obvious that the amount of hydroxyproline (fig 5) is correlated with the tensile strength (fig 1) in normal implants In samples from aminoacetonitrile-treated rats there was a discrepancy between the tensile strength and the synthesis of total hydroxyproline In normal implants the nitrogen content rose steadily during the first 15 days, but in aminoacetonitrile treated animals it persisted at 5th day level (fig 6)



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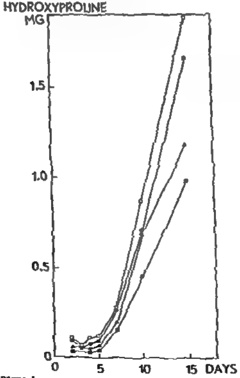

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[illegible] droxyproline (fig 5)

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tensile strength and the synthesis of total hydroxyproline [illegible] normal implants the nitrogen content rose steadily during the first 15 days, but in aminoacetonitrile treated animals it persisted at 5th day level (fig 6)

that the amount of nitrogen did not increase in the aminoacetonitrile treated rats as in the other groups, and most in the control group There seem no data available on the factors that regulate this protein accumulation before the collagen synthesis If the nitrogen were derived solely from passive imbibition with tissue fluids, there would not be so much difference between the groups Many explanations can be tentatively suggested for this effect of aminoacetonitrile (1) impairment in the growth of capillaries, thus restricting the blood flow and metabolic development in general (2) impairment of the division and proliferation of cells or (3) impairment of the synthesis of cellular proteins, with subsequent deficient maturation of the collagen synthesizing systems The incorporation of amino acids into collagen is decreased in lathyrism studied *in vivo* with guinea pigs (MARTIN, GROSS, PIEZ & LEWIS 1961) This could be explained by an effect on capillaries (which are weak in lathyrism), but the decreased collagen synthesis can be produced also in granuloma slices (KULONEN, SALMI & JUVA 1961) There is a general, but not understood, impairment of amino acid metabolism The similar effect of the "carbonyl fixatives" semicarbazide and iproniazid, may be due to their action on general metabolism

Up to a certain limit the growth of the granuloma depends on the nutritional stage of the animal (WILLIAMSON, MCCARTHY & FROMM 1951) In smaller animals the implant produces a larger strain on the animal, and the capillary growth also may be proportionally less

Summary

Administration of either aminoacetonitrile or semicarbazide decreased the tensile strength in developing experimental granulation tissue Similar, but probably less specific, effects were observed after administration of *iso*propyl*iso*nicotinic acid hydrazide

The weight and the nutritional state of the animals were important factors in the production of tensile strength in healing wounds and in granulation tissue

Normally the tensile strength in the control sponge implants increased in parallel with the amount of hydroxyproline in the granulation tissue In aminoacetonitrile treated animals the tensile strength developed slowly, but the production of hydroxyproline was not inhibited to the same degree The total nitrogen in the granulation tissue began to rise already before the collagen synthesis and most rapidly in the control samples In aminoacetonitrile treated animals the nitrogen content of the granulation tissue persisted at the 5th day level

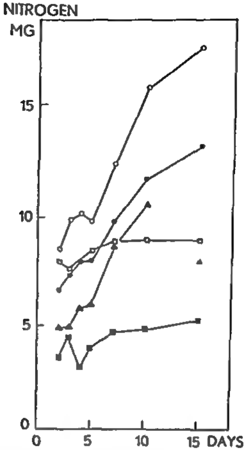

Fig 6 Total nitrogen per sponge implant at different times after implantation (○ control ● iproniazid 5 mg/100 g daily, ▲ semicarbazide 25 mg/100 g daily □ aminoacetonitrile 25 mg/100 g daily, ■ aminoacetonitrile 50 mg/100 g daily

Discussion

It was expected that aminoacetonitrile would cause a discrepancy between the amount of hydroxyproline and the tensile strength because of the increased solubility of collagen It is obvious that there developed little tensile strength in aminoacetonitrile treated rats (fig 1) The synthesis of hydroxyproline was retarded, but by no means completely inhibited (Fig 5) This is in agreement with the concept that in lathyrism the soluble forms of collagen do not mature into mechanically strong fibres The results for semicarbazide and iproniazid seem to lie between the control and that of aminoacetonitrile and may be partly non specific

Before the collagen synthesis begins in the sponge implants, there is a "lag phase" Already during that time the amount of nitrogen increased During the first days there occurred a passive imbibition by the sponge with tissue fluids and the immigration of cells The difference between various groups during these first days was not very large, but it is remarkable

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From the Department of Pharmacology, University of Helsinki

The Sedative and Lethal Actions of Reserpine in Mice, as Modified by 5-Hydroxytryptophan, 3,4-Dihydroxyphenylalanine, Methylphenidate, and Nikethamide in Cold and Warm Environments

By

M M Airaksinen and M Mattila

(Received May 12, 1962)

It has been demonstrated that reserpine does not decrease the spontaneous motility of mice if the body temperature is not decreased (LESSIN & PARKES 1957) GARATTINI & VALZELLI (1958) reported that reserpine administered after a cold stress of many hours caused no sedation and had no effect on the 5 hydroxytryptamine (5HT) content of rat brain At an environmental temperature of 37° reserpine did not increase barbiturate sleeping time and had no consistent effect on the amount of 5HT in the brain BRODIE *et al* (1960) reached almost the same results in their cold experiments (+4°), they also noted a greater fall in the norepinephrine than in the 5HT content of rat brain

CARLSSON *et al* (1957) reported that 3,4-dihydroxyphenylalanine (DOPA) alone, and to an even greater extent when used with 5 hydroxytryptophan (5HTP), abolished the sedative effect of reserpine *in mice* at room temperature DOPA also diminished the toxicity of reserpine in mice exposed to cold (NEČINA & KREJČI 1961)

The purpose of our work has been to investigate the effect of DOPA, 5HTP and some stimulants of the central nervous system on the sedative and lethal action of reserpine in mice at cold and warm environmental temperatures

Materials and Methods.

Male white mice weighing 17–25 g were kept at room temperature (23°) before the experiments During the experiment they were placed in plastic cages, five in each, at an environmental temperature of +9° or +37°

Reserpine (CIBA) dissolved in acetic acid, dl 5 hydroxytryptophan (Hoffmann La Roche) dl 3,4-dihydroxyphenylalanine (Hoffmann La Roche), methylphenidate

Acknowledgements

This work forms a part of a program supported by U S Department of Agriculture through its Foreign Research and Technical Programs Division H I acknowledges the personal grant from President Kekkonen's Foundation We also thank the Directors of Sateri Oy, Ltd Valkeakoski for their generosity in the preparing and supplying the viscose cellulose sponges

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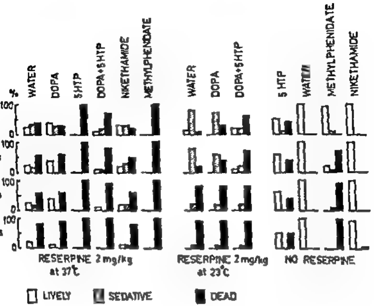


after being put into the warm environment Ten mice in each group

The mice receiving reserpine 2 mg/kg at room temperature were slightly more sedated than those receiving the drug in the cold Some mice received reserpine just at the time of being placed in the cold and showed the same effects as the previously cold stressed animals

5HTP increased the sedation and mortality of the reserpinized mice

DOPA at the doses used did not alter the 24-hour mortality In those groups in which reserpine induced sedation and mortality were apparent in 4–8 hours, these effects were antagonized by DOPA Smaller doses of this amino acid (30–100 mg/kg) had the same effect when administered repeatly at the same intervals When given along with DOPA, 5HTP prevented the stimulating action of DOPA

Nikethamide and *methylphenidate* were administered to the groups of mice receiving reserpine 2 hours before the cold exposure They reduced the sedative action and mortality due to reserpine Methylphenidate was the best antagonist to reserpine

Heat experiments (figure 2) All the control mice were alive after exposure to the environmental temperature of 37° for 24 hours Most of the reserpinized mice died under these conditions Convulsions were common, and the sedative action was never very pronounced Slight sedation was more common in the groups receiving reserpine at 23° before

hydrochloride (Ritalin ®, CIBA) and nikethamide (Corditon ® Medica) in aqueous solution were injected intraperitoneally

The reserpine dose was 2 mg/kg It was given either as a single dose or one mg per day on two consecutive days With the animals kept at a cold or a warm temperature some groups received 200 mg/kg DOPA or 5HTP, separately or together, three times at intervals or 3–4 hours Some groups received 50 mg/kg nikethamide or 25 mg/kg methylphenidate hydrochloride in the same way

Results

A *Cold experiments* The temperature of +9° (+6° to +11°) was used because all the control mice survived it for 24 hours At lower temperatures (0° and +4°) most of the mice died in some hours

The main results are seen in figure 1 After reserpine, administered as a single dose of 2 mg/kg a few hours before or after beginning the exposure to cold, all mice died within 24 hours About half of them died in 6 hours

The same results were also observed when 1 mg/kg of reserpine was administered twice, the second dose at the beginning of cold exposure If the last dose was given 24 hours previously, all the mice survived 6 hours and some 24 hours The sedative action was lowest in this group

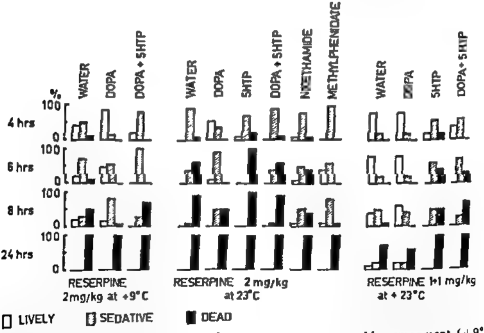
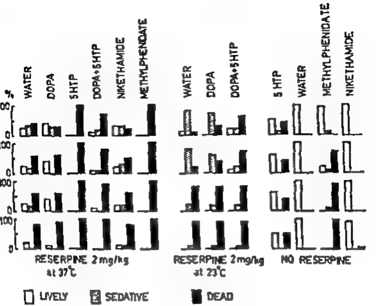


Figure 1 Sedative and lethal actions of reserpine to mice in cold environment (+9°), modified by various drugs Reserpine 2 (mg/kg) was injected as a single dose 2½ hrs ... cold or in two doses 48 and 24 hrs



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B *Heat experiments* (figure 2) All the control mice were alive after exposure to the environmental temperature of 37° for 24 hours Most of the reserpinized mice died under these conditions Convulsions were common, and the sedative action was never very pronounced Slight sedation was more common in the groups receiving reserpine at 23° before

beginning heat exposure than in those receiving it at 37° The survival time of the latter was somewhat shorter than that of the former animals

5HTP shortened further the survival time of the reserpinized animals This effect was more marked at 37° than at 9° When 5HTP was administered alone at 37°, it was lethal to half of the mice

DOPA alone has no clear effect on reserpine mortality at 37°, but it partly antagonized the effect of 5HTP in reserpinized mice

Methylphenidate stimulated the spontaneous motility and markedly decreased the survival time of both normal and reserpinized mice *Nikethamide* was almost inactive at this temperature

At a higher surrounding temperature (+39°) the toxicity of reserpine as well as that of 5HTP, increased further, but normal control mice remained lively Methylphenidate first increased motor activity and then soon caused the death of the reserpinized mice, as at 37° environmental temperature Nikethamide had no effect on the survival time

Discussion

The mortality of the reserpinized mice was high in both cold and warm environments GARATTINI & VALZELLI (1958) and BRODIE *et al* (1960 found no notable sedation in rats if reserpine was injected in the cold after a 4 hours exposure to cold Most of our mice, however, were sedated in cold experiments It is possible that mice are so little resistant to cold stress that the "sedation" is, at least in part, due to exhaustion, as emphasized by ERSPAMER (1961)

The less marked reactions in those mice receiving reserpine 24 hours before exposure to cold suggest that these effects are due to some other effects of reserpine rather than to the liberation of amines in the brain (SHEPPARD & ZIMMERMAN 1959) *This view is supported* by the fact that nikethamide and methylphenidate also diminished the effects of reserpine in the cold, although they have not been shown to cause depletion of the brain amines It is possible that DOPA, nikethamide and methylphenidate in our cold experiments acted as non-specific stimulants that increased motor activity They would then have increased heat production and antagonized the hypothermia caused by the paralysis of thermoregulation Possibly, then these drugs did not inhibit the action of reserpine in the heat experiments and the toxicity of reserpine was even enhanced by methylphenidate, the best antagonist in the cold and the strongest motor stimulant in the heat

Besides the central actions of reserpine, the depletion of catecholamines of the adrenal glands and other peripheral tissues may modify thermoregulation (HOFFMAN 1959) KRONEBERG & SCHUMANN (1960) have de-

monstrated a much more pronounced depletion of adrenaline from the adrenal medulla after reserpine in the cold than at the normal room temperature

Although reserpine toxicity in the heat environment was suprisingly high, sedative action was absent So the increased "free" 5HT (BRODIE 1957) need not be responsible for the lethal action of reserpine, although BRODIE *et al* (1960) considered this to be associated with the tranquillizing action of reserpine Because 5HT reduces the body temperature (FASTIER *et al* 1957), the increased reserpine toxicity after 5HTP in the cold and the heat experiments may have been due to superadded depressing of thermoregulation caused by reserpine and 5HT This possibility is supported by the observation (KARJA *et al* 1961) that in mice at room temperature 5HTP can, depending on the dose, decrease or increase the body temperature SHEMANO & NICKERSON (1959) reported that at environmental temperatures below 30° 5HT reduces the body temperature of the rat, but increases it at temperatures above 30°

As mentioned before, CARLSSON *et al* (1957) showed that DOPA and 5HTP together effectively antagonized the action of reserpine at room temperature In our experiments performed at higher and lower temperatures the reserpine antagonizing effect of DOPA was inhibited by 5HTP

It should be noted that 5HTP and DOPA have been observed to compete for the same decarboxylating enzyme (WESTERMANN *et al* 1958, YUWILER *et al* 1959) Further, an antagonism between 5HT and norepinephrine *in vivo* has been reported (GORDON *et al* 1958)

Summary

The effect of 5 hydroxytryptophan (5HTP), 3,4 dihydroxyphenylalanine (DOPA) nikethamide and methylphenidate on the sedative and lethal actions of reserpine has been studied in mice kept at cold (+9°) or warm (+37° and 39°) environmental temperatures

In the cold, a half of the reserpinized mice died in eight hours and all of them in 24 hours The sedative action in the cold was almost the same when reserpine was administered in the cold or before beginning the cold experiment If reserpine was given in two doses and the cold experiment was begun 24 hours after the second, the animals survived a little longer and showed less sedation DOPA, nikethamide, and methylphenidate decreased and 5HTP increased the sedative and lethal actions of reserpine, DOPA partly antagonized this action of 5HTP

In the warm environment most of the reserpinized mice died in 24 hours Convulsions were common, sedation was slight and infrequent Without reserpine 5HTP ... lethal to half the mice As in the cold, it greatly in-

creased the toxicity of reserpine. This effect of 5HTP was partly antagonized by DOPA; when given alone DOPA had no effect on the toxicity of reserpine.

Methylphenidate alone was very toxic in the heat. It also greatly enhanced the lethality of reserpine, whereas *nikethamide was inactive.*

Acknowledgements.

We are indepted to Ciba AG, Basel, for reserpine and methylphenidate

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On the Anticonvulsant Activity of Local Anaesthetics

By

H -H Frey

(Received May [illegible] 1962)

Local anaesthetics are generally regarded as convulsant drugs However, there have been some reports in the past decade about an anticonvulsant property of such drugs (GERLICH 1950, BERNHARD *et al* 1954/56, TANAKA 1955, KAPILA & ARORA 1962) These reports seem not to have received much attention, possibly because they were in part conducted by methods somewhat unusual in pharmacology

Nevertheless seemed it of interest to evaluate the anticonvulsant activity of some local anaesthetics by the usual methods and to compare their activities with those of known anti epileptic drugs, e g phenobarbital and diphenylhydantoin

Methods

All work was done on mice of both sexes of the Leo strain, weighing 20–22 g

The anticonvulsant activity was determined by the methods of SWINYARD (1949) and of GOODMAN *et al* (1953)

1 Maximal electro-shock seizure the endpoint for 'anticonvulsant action' being the abolition of the tonic extensor component, 2 the pentetrazole seizure threshold test The mice received a subcutaneous injection of 100 mg/kg pentetrazole (CD95) and were observed for 30 min Mice were considered protected if they did not show clonic convulsions within this time The thresholds for the clonic convulsion and the tonic extensor component were determined on mice by fractionated intravenous injection of a 0 5% solution of pentetrazole as in the method of ORLOFF *et al* (1949) Finally the neurotoxicity was determined as described by SWINYARD *et al* (1949, 1952) and [illegible]

test (ET50) by that of LITCHFIELD (1949) All doses are given in terms of the free base or acid

creased the toxicity of reserpine. This effect of 5HTP was partly a
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Methylphenidate alone was very toxic in the heat. It also grea
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Table 2.

Anticonvulsant activities of lidocaine and butamine in the maximal electro shock seizure test 15 min after application

Compound	Application	ED 50 mg/kg
Lidocaine	sc	11 (9 2–13)
	orally	23 (21–26)
Butamine	sc	12 (10 5–14 5)
	orally	23 (19–29)

Phenobarbital diphenylhydantoin and oxypolyethoxydodecane were given orally or subcutaneously one hour before application of the electro-shock or the subcutaneous injection of pentetrazole, the other local anaesthetics 30 min before electro-shock and 20 min before the subcutaneous injection of pentetrazole The anticonvulsive ED50 s of lidocaine and butamine in the electro-shock test were also determined 15 min after the oral or subcutaneous application The activities against electro-shock were finally determined 2 min after intravenous injection of lidocaine, butamine or tetracaine and 5 min after oxypolyethoxydodecane Neurotoxicity was determined one hour after the application of phenobarbital, diphenylhydantoin or oxypolyethoxydodecane and 30 min after the other local anaesthetics The ORLOFF-test was conducted one hour after phenobarbital and diphenylhydantoin 45 min after oxypolyethoxydodecane and 20 min after the other local anaesthetics

Results

The results are summarized in the tables 1–3 It can be seen from these tables that lidocaine and butamine had nearly the activity of the two reference compounds in the electro shock test 15 and 30 min, respectively after subcutaneous injection Orally applied they were less active, but still relatively potent The distance between the anticonvulsive and the neurotoxic dose effect lines compared well with those of phenobarbital and diphenylhydantoin, the difference between ED95 and TD5 being significant for lidocaine and diphenylhydantoin but not for butamine and phenobarbital The first sign of neurotoxicity of the local anaesthetics was usually diminished abdominal muscle tone The main difference between lidocaine and butamine, on the one hand, and the classical anticonvulsants, on the other, lay in their duration of action, which was about one hour for the local anaesthetics compared with 8 hours and more for phenobarbital and diphenylhydantoin

Tetracaine and the detergent oxypolyethoxydodecane showed some anticonvulsant activity only after subcutaneous injection in the range of neurotoxic doses, they were inactive after oral application Probably both compounds are slowly absorbed from the intestine, the high pK-value of

Table 1

Anticonvulsant activities of local anaesthetics, compared with those of phenobarbital and diphenylhydantoin

Compound (time)	Application	Electro shock ED 50 mg/kg	Neurotoxicity TD 50 mg/kg	TD 50/ ED 50	Duration of action		Pentetrazole sc ED 50 mg/kg	TD 50/ ED 50
					ED 95 mg/kg	ET 50		
Phenobarbital (1 h)	orally	17 (15–19)	33 (29–36)	1 9	23	~8 h	13 (10–16)	2 5
Diphenylhydantoin (1 h)	orally	11 (9 2–13)	100 (83–125)	9 1	18	>8 h	ineffective	
Lidocaine (30 min)	sc	15 (13–17)	62 (53–73)	4 1	20	63 min	ineffective	
	orally	29 (26–32)	120 (105–140)	4 1	37 5	50 min		
Butamine (30 min)	sc	18 (15 21)	41 (33–51)	2 3	23	59 min	ineffective	
	orally	22 (19–26)	75 (67–83)	3 4	36	53 min		
Tetracaine (30 min)	im	16 (13–18)	14 (12–18)	0 88	27	52 min	ineffective	
	orally	ineffective	88					
Oxypolyethoxydodecane (1 h)	sc	92 (75–110)	70 (59–83)	0 76	170	2 1 h	ineffective	
	orally	150 (partially effective)	110 (100–120)					

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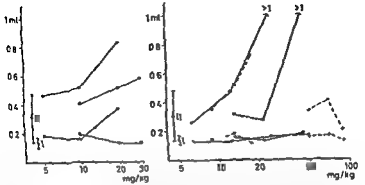


Fig. 1 Influence of pretreatment with phenobarbital diphenylhydantoin or local anaesthetics on the pentetrazole threshold for (I) clonic convulsions and (II) the tonic extensor component. Ordinate ml of 0 5 per cent pentetrazole solution per 20 g mouse
Symbols Left graph ●——● Phenobarbital ○——○ Diphenylhydantoin
Right graph ●——● Lidocaine ○——○ Butanilicaine ×----× Tetracaine
+----+ Oxypolyethoxydodecane

However, the clinical results of BERNHARD *et al* (1954, 1955) and BOHM (1958) have shown that intravenous lidocaine can be useful in status epilepticus, at least if the attacks are of grand mal or JACKSON type There appeared to be a similarity to diphenylhydantoin in that both only acted against electrically induced convulsions and not against pentetrazole-induced seizures, and it is well known that diphenylhydantoin also acts primarily against grand mal epilepsy

It is surprising that local anaesthetics did not in any way affect the pentetrazole threshold for clonic convulsions A lowering of this threshold would have been detected by the method of ORLOFF, since there are only about 4 sec between the intravenous injection of a convulsive dose of pentetrazole and the beginning of clonic fits One might conclude that pentetrazole treatment of an intoxication with local anaesthetics – which is generally held to be contraindicated on account of the convulsant qualities of both compounds – might not inevitably lead to an aggrevation of the clinical picture ZIPF & HOPPE (1936) have shown that there can be a detoxifying action of pentetrazole against local anaesthetics But this statement also seems of merely theoretical interest, especially since there is hardly any argument in favour of such a treatment

The mechanism of the central anticonvulsant action of local anaesthetics need *not* be different from the action on the peripheral nerve stabilization or even hyperpolarization of the nerve cell, which will prevent the spread of excitation potentials in the brain BERNHARD *et al* (1956) have shown that lidocaine abolished the cortical after discharge after electrical stimulation of the cortex in all regions of the cortex, and not only in those

Table 3

Anticonvulsant activities of local anaesthetics in the maximal electro shock seizure test after intravenous injection

Compound	Time of testing after iv injection	ED 50 mg/kg
Tetracaine	2 min	0 56 (0 51–0 62)
Lidocaine	2 min	2 (1 5 2 8)
Butamine	2 min	4 (3 5–4 7)
Oxypolyethoxydodecane	5 min	16 (15 18)

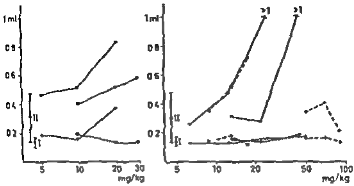
tetracaine (8 33 according to BUCHI & PERLIA 1960) being in favour of this possibility It is uncertain if oxypolyethoxydodecane passes the blood brain barrier readily Procainamide, being inactive as a local anaesthetic showed no anticonvulsant activity in subtoxic doses (up to 75 mg/kg sc)

After intravenous injection the anticonvulsant activity of the local anaesthetics roughly paralleled their local anaesthetic effect (tab 3) oxypolyethoxydodecane again lagging behind

Like diphenylhydantoin, all local anaesthetics were ineffective against the clonic convulsions evoked by subcutaneous injection of pentetrazole This similarity appeared also in the ORLOFF test (fig 1) after pretreatment with phenobarbital the thresholds for clonic convulsions (I) as well as for the tonic extensor component (II) were elevated whereas with diphenylhydantoin this was only true of the second one After pretreatment with local anaesthetics the threshold for clonic convulsions remained unaltered over the whole dose range but that for the tonic extensor component was markedly raised by lidocaine, tetracaine and butamine and finally this phase of the convulsion was abolished Oxypolyethoxydodecane again behaved differently

Discussion

Our results confirm earlier reports on the anticonvulsant property of local anaesthetic agents and seem to call for some revision of current concepts about the convulsant nature of these compounds At any rate some of the common local anaesthetics have a strong anticonvulsant activity at a dose range well below convulsant (toxic) doses This statement seems at present to be of more theoretical than practical importance since it is thought that local anaesthetics have generally too short a duration of action to guarantee a steady anticonvulsant blood and brain level of drug and that the local anaesthetic activity is besides an undesirable side-effect



Right graph ●——● Lidocaine ○——○ Butamine, × - - - - × Tetracaine, + - - - - + Oxypolyethoxydodecane

However, the clinical results of BERNHARD *et al* (1954, 1955) and BOHM (1958) have shown that intravenous lidocaine can be useful in status epilepticus, at least if the attacks are of grand mal or JACKSON type There appeared to be a similarity to diphenylhydantoin in that both only acted against electrically induced convulsions and not against pentetrazole-induced seizures, and it is well known that diphenylhydantoin also acts primarily against grand mal epilepsy

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remote from the locus of stimulation as happens with the barbiturates A certain local anaesthetic potency seems to be necessary for anticonvulsant effect, procainamide, being devoid of local anaesthetic activity, showed only at highly toxic doses any effect against electro shock

The weak anticonvulsant action of oxypolyethoxydodecane in relation to its local anaesthetic potency could be the consequence of too slow a passage through the blood-brain barrier. Could, then, the antitussive activity of this compound, nearly as powerful as that of codeine (GOTTFRIEDSEN 1959), be of predominantly peripheral nature and related to the actions of this compound on vagal afferences (ZIPF & KREPPEL 1955, ZIPF & REICHERTZ 1957)?

Summary

The anticonvulsant activities of the local anaesthetics lidocaine buta mine, tetracaine and oxypolyethoxydodecane have been determined in mice and compared with those of phenobarbital and diphenylhydantoin In the electro-shock test lidocaine and butamine showed activities of the same order of magnitude as those of the reference compounds, but of much shorter duration Like diphenylhydantoin, the local anaesthetics were ineffective against clonic convulsions evoked by subcutaneous injec tion of pentetrazole

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From the Anesthesia Laboratory of the Harvard Medical School
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Influence of Hypercarbia on the Activity of Neuromuscular Blocking Agents in the Cat

By

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(Received May 28, 1962)

In cats PAYNE (1958, 1959, 1960 a, 1960 b, 1961), using a divided dose technique, investigated the effects of induced respiratory acidosis and "metabolic" acidosis and alkalosis on the activity of neuromuscular blocking agents and found that gallamonium, decamethonium, suxamethonium, benzoquinonium and dimethyl tubocurarine were each antagonized by carbon dioxide That the effect of (+) tubocurarine was enhanced by this gas was ascribed to the fact, originally noted by KALOW, that only (+) tubocurarine has a pH value within the physiological range and thus is susceptible to the changes in dissociation that occur with changes in the acidity of the blood

Later experiments did not support this view, (PAYNE 1960 b) since a decrease in the activity of (+) tubocurarine was demonstrated when acidosis was induced by infusion of 0 1 N hydrochloric acid

GAMSTORP & VINNARS (1961) found in rabbits that the effect of acidosis and alkalosis on neuromuscular transmission was neglible and that the activity of (+) tubocurarine increased in acidosis, whether this was induced by carbon dioxide or by acid infusion, and decreased in alkalosis

(+) Tubocurarine stands out from the other myoneural blocking agents in common use in having also a blocking action on the sympathetic ganglia not far below that of hexamethonium (PATON 1959) Hypercarbia is considered a strong stimulant of the sympathetic nervous system (FENN & ASANO 1956), and the importance of the sympathetic mediators for the function of striated muscle is recognized (GOFFART & RITCHIE 1952, NAESS & SIRNES 1953, BOWMANN & ZAIMIS 1958, ISAACS *et al* 1960)

*) Present address Copenhagen County Hospital, Gentofte, Hellerup, Denmark

In our investigation respiratory acidosis was induced in the presence of neuromuscular blockade and the sympathetic outflow was blocked epidurally during the procedure as well

Technique

Cats were anaesthetized with pentobarbital sodium (mebumal sodium (NFN) = nembutal ®) 60 mg/kg i m and tracheotomized, and the carotid artery and jugular vein were cannulated for blood pressure recording and infusion, respectively Controlled respiration was used through all the experiments, either with atmospheric air or with 10% CO_2 in O_2 The intravenous infusions of neuromuscular blocking agents were given continuously by way of an infusion pump For sympathetic blockade, two plastic catheters were introduced into the epidural space, and 0 5% procaine was injected intermittantly until a stable level of hypotension was reached, as described by BREWSTER et al (1952)

The sciatic nerve was cut, and the peripheral end was stimulated with supramaximal square wave stimuli of 1 msec duration and a frequency of 1 per 2 sec The corresponding leg was firmly fixed and isometric contractions were recorded with a S_4 type Walton strain gauge to which the tendons were tied The recordings were made on a Sanborn Polygraph

The standard procedure was as described below Once a steady level of curarization had been establ

Results

Neuromuscular blockade was produced by the infusion of suxamethonium, decamethonium, gallamonium or (+) tubocurarine The effect of carbon dioxide on the neuromuscular blockade produced by these agents was slight, manifesting itself as gradual changes in the twitch tension at the onset and discontinuation of the carbon dioxide

Determinations of pH and pCO_2 in the arterial blood of 10 cats showed the pH before exposure to carbon dioxide to average 7 35 (max 7 43, min 7 24) and pCO_2 35 3 mm Hg (max 50, min 13 mm), pH after 30 min of exposure to carbon dioxide averaged 6 97 (6 89–7 13) and pCO_2 99 4 (68–132)

The infusion rates averaged

suxamethonium chloride	15	µg/kg/min
decamethonium iodide	0 7	µg/kg/min
gallamonium iodide	60	µg/kg/min
(+) tubocurarine chloride	3	µg/kg/min

Suxamethonium A group of 5 cats were exposed to carbon dioxide a total of 8 times In one cat antagonism was displayed on both muscles

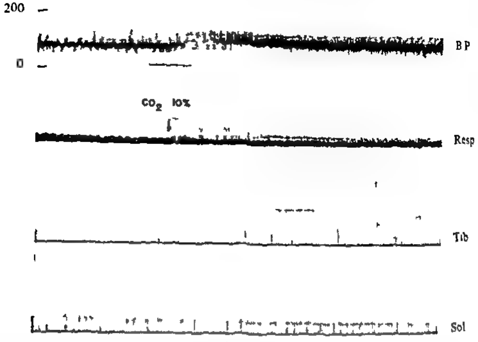

Fig 1 The effect of induced respiratory acidosis on the neuromuscular blocking effect of gallamonium in the cat Gallamonium is continuously infused at a constant rate and the twitch tensions of the tibial (Tib) and soleus (Sol) muscles are depressed more than 50 % At arrow, 10% CO_2 in O_2 is introduced as ventilatory air A weak antagonistic response is observed The respiration (Resp) was controlled but the carbon dioxide evoked spontaneous respiratory movements and produced a rise in the blood pressure (B P)

within the first 5 minutes of the exposure In another cat, a similar response was observed on the soleus muscle In 11 instances there was no response, and two responses could not be evaluated On discontinuing the CO_2, no cat showed a decrease in neuromuscular block, but in two cats the block increased in both muscles and in one the tension of only the soleus dropped

Three cats were given an epidural sympathetic block during their second exposure to carbon dioxide, which produced a slight decrease in tension of the twitch, but all showed an increase in twitch tension when the carbon dioxide was turned off

The *blood pressure* response to carbon dioxide was an immediate rise After the sympathetic block, which lowered the blood pressure 30–40% the blood pressure rose 10–20 mm on turning off the carbon dioxide

Decamethonium A group of four cats were exposed to carbon dioxide a total of 8 times One developed antagonism on both muscles within the first 5 minutes, and antagonism was demonstrated on two tibial muscles and two soleus muscles without response of the other muscle No response was elicited in 3 cats No instance of potentiations with CO_2 was found On discontinuation of the CO_2, the contractions decreased in one cat, remained unchanged in one and increased in another This last cat was

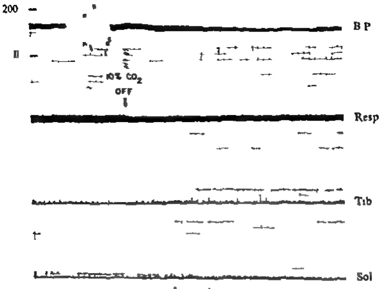

Fig 2 The eff... nuously soleus (... arrow af... is obser...

submitted to two exposures of carbon dioxide before the sympathetic block and on both occasions increased twitch tension developed after removal of carbon dioxide Epidural sympathetic block was performed in 3 of the cats during exposure to CO_2 and in each one an increase in contraction upon withdrawal of carbon dioxide was observed in both muscles

The *blood pressure* rose instantaneously after the administration of carbon dioxide it decreased during the sympathetic block to 30–40% of control levels and increased after discontinuation of carbon dioxide in these animals

Gallamonium Four cats were exposed to carbon dioxide (figure 1) The first three were exposed 5 times and antagonism was displayed in all cats on both muscles Af...
twitch tension increa...
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nistered and here the ... of carbon dioxide was one of dep...

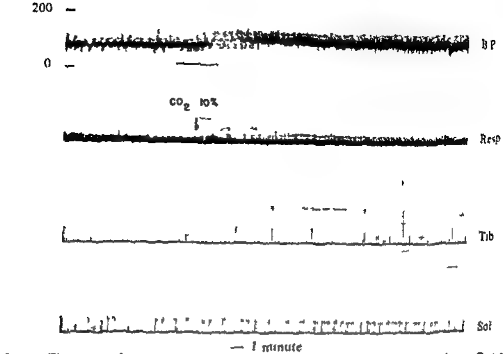

Fig 1 The effect of induced respiratory acidosis on the neuromuscular blocking effect of [illegible]

response is observed The respiration (Resp) was controlled, but the carbon dioxide evoked spontaneous respiratory movements and produced a rise in the blood pressure (B P)

within the first 5 minutes of the exposure In another cat, a similar response was observed on the soleus muscle In 11 instances there was no response, and two responses could not be evaluated On discontinuing the CO_2, no cat *showed a decrease in neuromuscular block*, but in two cats the block increased in both muscles, and in one the tension of only the soleus dropped

Three cats were given an epidural sympathetic block during their second exposure to carbon dioxide, which produced a slight decrease in tension of the twitch, but all showed an increase in twitch tension when the carbon dioxide was turned off

The *blood pressure* response to carbon dioxide was an immediate rise After the sympathetic block, which lowered the blood pressure 30–40%, the blood pressure rose 10–20 mm on turning off the carbon dioxide

Decamethonium A group of four cats were exposed to carbon dioxide a total of 8 times One developed antagonism on both muscles within the first 5 minutes, and antagonism was demonstrated on two tibial muscles and two soleus muscles without response of the other muscle No response was elicited in 3 cats No instance of *potentiations with CO_2 was found* On discontinuation of the CO_2, the contractions decreased in one cat, remained unchanged in one and increased in another This last cat was

is the only variable With this technique the variations in activity of the myoneural blocking agents induced by carbon dioxide were on a much smaller scale than those of Payne's This may be because the increase in tissue concentrations of carbon dioxide was gradual (NICHOLS 1958) It appears that the changes in neuromuscular blockade produced by 10% carbon dioxide over a 30 minute period, or longer, are undramatic, even when the degree of respiratory acidosis developed is severe

The responses were more clear-cut with the non depolarizing agents than with suxamethonium and decamethonium, but qualitatively they responded in the same direction as in Payne's experiments, apart from the one cat in the decamethonium group that showed increase in contractions upon withdrawal of carbon dioxide

The reason for the delayed rise in blood pressure, preceded by an initial fall, upon administration of carbon dioxide during infusion of (+) tubocurarine, is probably found in the ganglion-blocking effect of the drug Epidural sympathetic blockade was performed in one group of cats while they were under the influence of the neuromuscular blocking agent and carbon dioxide The blood pressure rarely dropped below [illegible] mm Hg and was above this level when the carbon dioxide was discontinued The twitch tension would decrease slightly as the block took effect Upon discontinuation of carbon dioxide, in these circumstances, an increase in twitch tension was invariably observed, indicating that carbon dioxide had enhanced the activity of all the agents

The fact that epidural blockade of the sympathetic outflow removes the discrepancy between the effect of increased inspiratory tensions of carbon dioxide upon (+) tubocurarine and the other muscle relaxants, suxamethonium decamethonium and gallamonium, suggests that the ganglionic blocking effect of (+) tubocurarine, by modifying the release of catecholamines, is a contributory factor in creating these differences The increase in sympathetic activity in the presence of carbon dioxide might contribute to the augmented muscular activity present when the muscles were under the influence of the other muscle relaxants

No difference was observed in the reaction of the tibial and the soleus muscle either qualitatively or quantitatively in these experiments

Summary

In cats respiratory acidosis was induced by means of ventilation with 10% carbon dioxide in the presence of partial neuromuscular blockade The effect on the myoneural block was slight, but a weak antagonism was observed with suxamethonium, decamethonium and gallamonium, whereas the effect of (+) tubocurarine was enhanced

Tubocurarine . (+) Tubocurarine was infused into 6 cats, the total number of exposures to carbon dioxide was 10 (figure 2) At the onset of CO_2 exposure, depression of the twitch tension occurred 6 times in both muscles, one time only in the tibial and one time only in the soleus muscle One cat did not show any response to carbon dioxide at the onset on two occasions Upon discontinuation of carbon dioxide, increase in twitch tension was observed in all cats Sympathetic block was performed on two of the cats during exposure to CO_2, and in both twitch tension increased when carbon dioxide was discontinued

The *blood pressure* response to carbon dioxide differed from that of the cats in which neuromuscular blockade was produced by suxamethonium decamethonium or gallamonium, by showing an initial decrease of 10-20 mm Hg and rising considerably more slowly

The sympathetic block *per se* produced a slight decrease in twitch tension in all animals

Discussion

PAYNE (1958) demonstrated altered responses of neuromuscular blocking agents after increased concentration of carbon dioxide in the inspired air The responses were elicited with 5, 10 and 20% CO_2 and they were quantitatively expressed as percentage depression of twitch tension developed before, during and after exposure to carbon dioxide The responses to suxamethonium, decamethonium, gallamonium and dimethyltubocurarine were diminished by carbon dioxide, whereas the paralysis produced by (+) tubocurarine was enhanced In seeking an explanation for these results, PAYNE (1958) discussed the possibility that carbon dioxide acts as an anticholinesterase and the influence of carbon dioxide on renal function, but he found no relationship between these factors and the results obtained and no correlation with the concomitant rise in serum potassium during carbon dioxide administration

It is especially surprising that acidosis antagonizes the effect of suxamethonium, in view of the fact that the optimal pH of the serum cholinesterase is 8 5 (AUGUSTINSSON 1948)

No explanation can at present be offered for the changes observed in the reaction of neuromuscular blocking agents in the presence of induced acidosis and alkalosis (PAYNE 1960 a) but (+) tubocurarine is the only relaxant investigated and differs in its mode of reaction from the other agents

The advantage of a continuous infusion technique is that, provided the infusion rate is constant and produces a constant level of muscular depression, the potential interactor, here carbon dioxide, can be introduced

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From the Research Laboratories of Draco, Lunds Farmaceutiska AB, Lund Sweden

Inhibition of Pepsin Proteolytic Activity by Some Common Antacids

By

Kaj Ö U. Persson and Björn Bunke

(Received June [illegible] 1962)

Gastric juice excerts its proteolytic action in the acid pH range It is a common belief that this action is always maximal between pH 1 and 2 and that the proteolytic activity at pH values higher than 4 is low or absent However, if the proteolytic activity of gastric juice is examined with different substrates and at different pH values it is evident that many proteins are digested with considerable speed at pH 3–5, e g casein, lactalbumin, ovalbumin, edestin, gliadin, gelatin, clupein and haemoglobin (Buchs & Freudenberg 1951, Merten & Ratzer 1949, Merten Ratzer & Kleffner 1949) Thus, the course of the activity-pH curve is dependent on the digested substrate It is of interest in this connection that the degree of denaturation of the substrates also seems to influence the course of the activity-pH curves (Linderstrøm Lang *et al* 1938; Bovey & Yanari 1960) The fact that gastric juice often possesses considerable proteolytic activity at pH values between 3 and 5 (and sometimes even shows maximum activity there) has caused discussions on the possible occurrence in gastric juice of a cathepsin besides pepsin*) (Bovey & Yanari 1960 [illegible] pepsin or another enzyme The most interesting substrate from a physiological and pathological point of view [illegible] the gastric mucosa, which is autodigested maximally at pH 4 5 according to Hayakawa (1951) Wound protecting coagulum will probably also be appreciably digested at pH 3–5 an assumption supported by the work of Linderstrøm Lang *et al* (1938) and Christensen (1955), which show that the pH maximum of digestion is displaced towards higher pH values when the substrates are denaturated As coagulum consists of non living protein, it [illegible] easily

*) Proteolytic activity between pH 1 and 3 is often defined as "pepsin activity" and proteolytic activity between pH 3 and 5 as "cathepsin activity"

When epidural sympathetic blockade was performed during the exposure to carbon dioxide, the block was slightly increased with all agents.

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Methods

Inhibition experiments These were performed as described below There were transferred 10 0 ml pepsin solution of the required concentration (in most cases 0 1–0 2 g/l), with or without NaCl and KCl (0 12 mole NaCl and 0 012 mole KCl/l), to a 50 ml jacketed beaker and it was allowed to equilibrate at 37 0°C for 5 min The experiments were performed with continuous stirring The concentrations of NaCl and KCl are the average ones reported to occur in gastric juice (HOPPE-SEYLER/THIERFELDER 1953) Immediately after the equilibration period the antacid was added gently The pH of the solution was not permitted to rise above 4 5 during the addition, to avoid irreversible inactivation of the pepsin due to too high a pH value The antacid was allowed to act for 10 min at the desired pH value, which had previously been adjusted on the pH-stat (pre-incubation) The pH value was then adjusted to that desired for the digestion, which was begun by adding 2.00 ml 10% (w/v) solution of the substrate The digestion period was exactly 5 min At the end of this period 6 0 ml of the digestion mixture were transferred to a flask containing 10 0 ml cold 4% (w/v) trichloroacetic acid After filtration the tyrosine liberated during the digestion was analysed colourimetrically, essentially as described by ANSON & MIRSKY (1933) Cf. FOLIN & CIOCALTEAU (1927) Modifications The extinction of the colour formed was measured in a spectrophotometer at 700 mμ, the corresponding amount of tyrosine being obtained from a standard curve based on pure tyrosine

Definition The proteolytic activity was expressed as mg tyrosine (properly tyrosine and substances equivalent to tyrosine by the method of analysis) liberated/ml digestion mixture the digestion period in minutes, as stated above

For every inhibition experiment a determination of enzyme activity without antacid was carried out (the "normal activity") This "normal activity" was determined under conditions identical with those of the inhibition experiments, both in pH and time schedule Thus the "normal activity" experiments were also subjected to 10 min pre-incubation at the same pH and temperature used for pre-incubation with antacid in the inhibition experiments Further, the same volume of liquid was added as had been injected by the titrator during the inhibition experiments. Blank experiments (salt solution or distilled water instead of pepsin or substrate) were performed in every series of experiments In the blank experiments 0 003–0 004 mg tyrosine/ml min were liberated These values were constant provided that the exper-

... solutions the pH of the digestion mixtures increased It fell to the desired value within 12–15 seconds from zero time If necessary, the acid input was increased manually Th...

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The volume of the syringe burette was 2 5 ml, and the concentration of the hydrochloric acid used was 1M

The error of a single determination of proteolytic activity was calculated to be at most ~7% It was considered to involve errors of dilution (±1%), weighing (±1%), time (±2%), pH (±0·1%) and spectrophotometry (±2%) The temperature fluctuations (±0 01°C) did not affect the results

denaturated by the acid gastric juice. Thus, gastric mucosa, and probably coagulum also, are not protected against digestion merely by neutralization of the "free acidity" of gastric juice (increase in pH to 3–4) As the pH values obtained by most modern antacids range from 3 to 5, it is of importance for the beneficial action of these substances that they should be able to reduce the digestion of a weakened (denaturated) gastric mucosa by inhibition of proteolytic activity.

The investigations quoted above and the conclusions drawn from them led us to investigate the ability of some common antacids to inhibit the cathepsin and pepsin activities of some commercial pepsin preparations *in vitro*. According to MERTEN & RATZER (1949) all commercial pepsin preparations possess considerable cathepsin activity. This was true of the preparations used in our investigation. Earlier investigations into the inhibition of the enzyme activity of gastric juice have essentially dealt with the activity at pH values less than 2.5 and only incompletely with the pH dependence of the inhibition. (KOMAROV & KOMAROV 1940; MARTIN & WILKINSON 1946; PIPER & FENTON 1961; SCHIFFRIN & KOMAROV 1941)

Experimental

Materials.

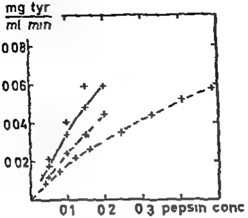
Enzymes and substrates *Pepsinum conc* "1 3000" (Parke, Davis & Co, London) CO-haemoglobin was prepared by the method of ANSON & MIRSKY (1933), with the modification that outdated human blood from a blood bank was used as raw material instead of ox-haemoglobin *Bovine plasma albumin fraction V* (Roussel Laboratories Ltd., London) Casein as described by HAMMARSTEN (Merck, Darmstadt) *Albumen ovi siccum*, Erg B 6 (Merck, Darmstadt)

Antacids Aluminum glycinate (Kaylene Chemicals Ltd, London) Aluminum hydroxide, dried gel, U S P XV, type F-1000, F-2000 and F-2200 (Reheis Comp Inc New Jersey, U S A) Aluminum hydroxide-magnesium carbonate, co-dried gel, type F-MA 11 (Reheis Comp) Bismuth subcarbonate, Swed Pharmacopaeia Ed XI (Distra, Stockholm) Calcium carbonate, Swed Pharm Ed XI (Distra) Calsil ®, calcium silicate (Astra, Sodertälje, Sweden) Dimagnesium aluminum trisilicate (K Thomae GmbH, Biberach a d Riss, Germany) Magnesium glycinate (Kaylene Chem) Magnesium oxide *pond*, Swed Pharm Ed XI (Distra) Magnesium perhydrol-pulver ®, magnesium peroxide (Merck, Darmstadt) Magnesium subcarbonate *pond*, Swed Pharm Ed XI (Distra) Magnesium trisilicate, U S P (J T Baker, Phillipsburg, N J. U S A),

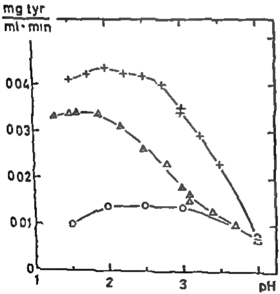
All other chemicals were of A R quality

Instruments "Titrigraph SBR2c", "Titrator TTT1c" and "Syringe Burette SBU1a", together constituting the pH-stat (Radiometer, Copenhagen) "Beckman Model DU Spectrophotometer" (Beckman Instruments, London) "Ultrathermostat NB- 30181" (Colora GmbH, Wurttemberg, Germany)

Volumes up to 3 ml were measured in calibrated constriction pipettes of the *Carlsberg* type and larger volumes with calibrated *Voll* pipettes Adequate corrections of the volumes were made at measurements of solutions whose temperatures diverged from room temperature.



2 The activity of a pepsin sample (expressed as mg tyrosine liberated/ml min) ... concentration (expressed as grams of pepsin per litre) Substrate ... ion temp 37°C Digestion pH 1 5, ... tion mixtures were 0 1 molar in NaCl and ... s represented by the broken line (no salts added) See methods for details



ig 3 Activity of a pepsin sample (expressed as mg tyrosine liberated/ml min) as a ...

See methods for details

It is important in experiments on enzyme inhibition that the ratio between the enzyme and the substrate concentrations is such as to allow performance of the experiments on the straight part of the curve representing the function *enzyme activity* = f · (*substrate concentration*) This meant that the highest enzyme activity (without inhibitor) available to us was 0 02 mg tyrasine/ml min for experiments *without* NaCl and KCl and 0 04 mg tyr /ml min for experiments *with* NaCl and KCL (see fig 2)

Recording of the pH time curves These curves were determined mainly as described by BEEKMAN (1960), 1 g antacid being dispersed in 15 ml distilled water and poured into 135 ml "artificial gastric juice" (0 032 M-HCl containing 2 g NaCl and 2 g pepsin 1 3000/l) at zero time Simultaneously, continuous injection of "artificial gastric juice" was begun The injection rate was maintained at 2 ml/min throughout pH was recorded continuously and with constant stirring The volume was kept constant by suction The temperature was 37°C

Methodology

In this kind of investigations it is important that the pH of the digestion and pre incubation mixtures be kept constant By using the pH stat this was easily achieved. The use of strong buffer solutions, which might have disturbed the reactions was also avoided

Haemoglobin and plasma albumin were used as substrates because their solutions were clear at all pH values used (pH 1–5) Other substrates investigated (casein oval bumin, and lactalbumin) gave rise to aggregating precipitates which caused variations in the experimental results

Other preliminary experiments included investigations into the significance of the pre incubation time for inhibition (see fig 1) determination of the connection between proteolytic activity and enzyme concentration (see fig 2) and control of the course of

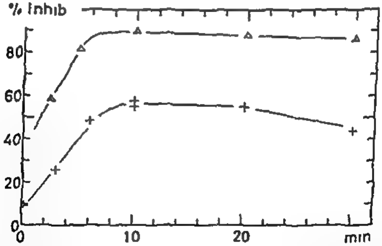


Fig 1 *Inhibition of a pepsin sample by aluminum hydroxide magnesium carbonate co dried gel* Per cent inhibition plotted against minutes of pre incubation at pH 4 0

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Table 1

Inhibition of the proteolytic activity of commercial pepsin preparations by some antacids The antacids were pre incubated with the pepsin solutions for 10 min at 37 C and pH 4 0 [illegible]

KCl per litre

Antacid	Per cent inhibition	Cf figure
Aluminum hydroxide magnesium carbonate co dried gel	58	4
Aluminum glycinate	43	5
[illegible]	27	4
[illegible]	25	5
[illegible]	20	6
[illegible]	7	6
[illegible]	4	–
Calcium carbonate	0	7

The pH dependence of the inhibition was determined for the most potent of the inhibitors (according to tab 1) and is illustrated in figs 8 and 9 The courses of the curves obtained are essentially independent of the substrate used for determining the enzyme activity See fig 8 This is because the enzyme activity without inhibitor (the "normal activity") was determined at the same pH values as the activity with inhibitor (see methods) As the inhibition is strongly dependent on pH, the inhibiting

Table 2

See the legend of tab 1 The antacids (except bismuth subcarbonate) were allowed to dissolve completely before the determination of the enzyme activity at pH 1 5

Antacid	Per cent inhibition	
	Digestion pH	
	3 1	1 5
Magnesium glycinate	90	90
[illegible]	[illegible]	85
[illegible]	65	65
[illegible]	58	0
[illegible]	43	1
[illegible]	27	+1
[illegible]	25	12
[illegible]	20	6

the activity pH curve (see fig 3) On the basis of the preliminary results the experimental method was designed

An objection to the design of the experiments is the possibility that the inhibition might be due to adsorption of liberated tyrosine on the antacids It was shown by experiments in which the substrate solutions had been substituted for tyrosine solutions that this objection was not valid

It is of importance for an adequate interpretation of the results of an investigation of this kind that the *in vitro* conditions resemble the *in vivo* conditions as closely as possible Thus temperature pH conditions concentrations of salts enzymes and other organic substances and of inhibitors should be kept at the same level in both Reproduction of temperature salt concentrations and pH conditions involved no problems It was however not always technically possible to maintain the same high enzyme activity *in vitro* as *in vivo* For that reason the experiments were performed with the same ratio between enzyme activity and antacid concentration as one would expect *in vivo* after antacid intake Information about the volume and enzyme activity of the gastric contents was obtained from a paper by MERTEN (1950/51) In this paper the enzyme activity of interdigestive secretion was stated to be 0 150 mg tyrosine nitrogen liberated/ml min (− 1 94 mg tyrosine/ml min) at pH 3 1 and the volume to be 20 ml If 1 g antacid is taken the resulting antacid concentration is 50 mg/ml and the ratio between enzyme activity and antacid concentration is 0 04 Thus comparison between the inhibiting ability of different substances was always made at an enzyme activity/antac conc ratio of 0 04 the dynamics of secretion being neglected The above applies to experiments at pH 3 1 When inhibition experiments were performed at other pH values if the enzyme *activity* was materially different (see figs 3 and 2 cf fig 1) the enzyme *concentration* was kept the same as in the experiments at pH 3 1 Therefore the ratio enzyme act /antac conc changed but the ratio between enzyme *concentration* and antacid concentration was the same as in the experiments at pH 3 1 At low enzyme activities (high pH values) the enzyme conc and antacid conc were increased proportionally

Results and Discussion

The results of the preliminary inhibition experiments are given in tab 1 Aluminum hydroxide magnesium carbonate co dried gel was the most potent inhibitor of proteolysis Strongly alkaline magnesium compounds have been omitted (see fig 12) being unsuitable as antacids because of the risk of rebound secretion (BRODY & BACHRACH 1959) The inhibition by these magnesium compounds was due to irreversible alkaline inactivation of the enzyme This was shown by experiments including pre incubation at pH 4 0 and determination of the enzyme activity at pH 1 5 after the antacids had been dissolved completely It appears from tab 2 that only the strongly alkaline substances destroyed the enzyme activity irreversibly under these conditions Evidently the pH values of these antacid grains immediate surroundings are considerably higher than indicated by the pH meter readings thus making alkaline destruction possible Alkaline destruction of the enzyme caused the results of the inhibition experiments to be poorly reproducible

Table 3

Percentage inhibition calculated from the "pH time" curves (Figs 10 and 11) and the "percentage inhib pH" curves (figs 8 and 9) The time interval between the first and second figures was about 100 minutes

Antacid	Percent inhib	Pre inc and digest pH
[illegible]	85–90	4 15–3 9
	80–60	4 1 –3 0
	75–55	3 9 –3 4
	20–35	1 7 –2 5
	20	1 9
Bismuth subcarbonate	10–15	1 7

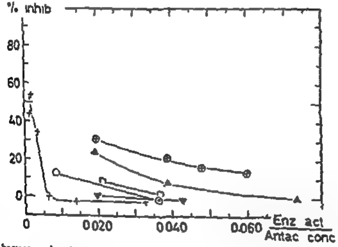


Fig 6 Abscissa and ordinate as ... cate with NaCl and KCl *Cir* ... NaCl and KCl. *Crosses* Do ... with NaCl and

antacids' ability to inhibit proteolytic activity This is true because the activity pH curves show different courses when the enzyme acts on different substrates (Cf fig 3) Thus, an antacid that gives rise to a pH value of 3 and inhibits the proteolytic activity by 50% at this pH value evidently gives better protection against proteolysis than a substance that gives rise to pH 5 and inhibits by 80% there if the substrate is digested most rapidly at pH 5 and very slowly at pH 3 It is not possible to elucidate the importance of this factor exactl...

... of human gastric mucosa (obtained by biopsy from

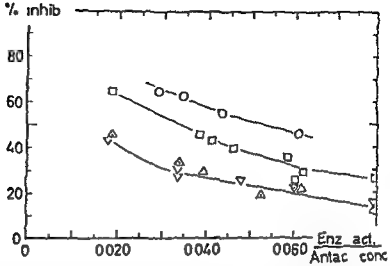


Fig 4 Percentage inhibition as a function of the ratio between enzyme activity without inhibitor and antacid conc Pre incubation performed at pH 4 [illegible] and digestion at pH 3 1 Temperature 37°C Enzyme activity without inhibitor 0 033 mg tyr liberated/ml min Enzyme conc 0 1 and 0 2 g per l with and without 0 1 mole NaCl and 0 01 mole KCl per l respectively Substrate 1 6% (w/v) CO hemoglobin
Circles Aluminum hydroxide magnesium carbonate co dried gel with NaCl and KCl *Squares* Do without salts *Triangles* Aluminum hydroxide dried gels F 1000 F 2000 and F 2200 The same curve applies to results with and without NaCl and KCl

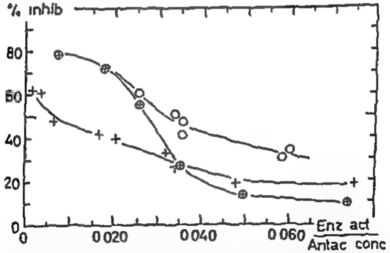
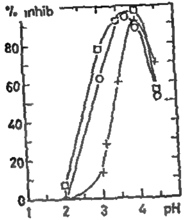


Fig 5 Abscissa and ordinate as in fig 4 *Circles* Aluminum glycinate with NaCl and KCl *Crossed circles* Do without salts *Crosses* Bismuth subcarbonate The same curve applies to results with and without NaCl and KCl

powers of the different antacids were compared at the pH values obtained by these antacids (see tab 3, and figs 8–12) Aluminum hydroxide magnesium carbonate, co dried gel was the most potent inhibitor under these conditions also

The substrate is the third factor to be considered at assessing the



[illegible] vs pH (= pre incubation pH) En-

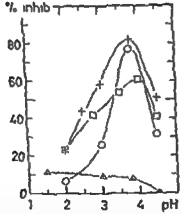


Fig. 9 Abscissa and ordinate as in fig 8 Substrate 1 6% (w/v) CO hemoglobin *Crosses* Aluminum glycinate *Circles* Dimagnesium aluminum trisilicate *Squares* Aluminum hydroxide, dried gel, F-2200 *Triangles* Bismuth subcarbonate

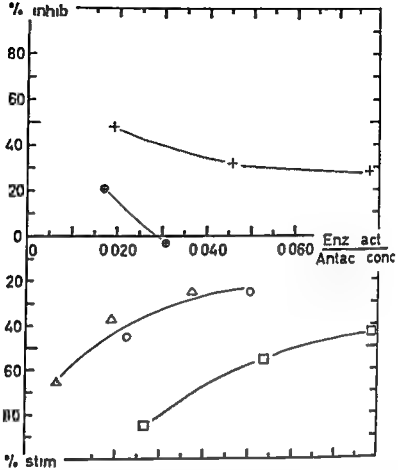


Fig 7 Abscissa and ordinate as in fig 4 In calculation of the quotients the weights of the salts were substituted for equivalent amounts of the corresponding oxides (aluminum and magnesium) or carbonates (calcium) *Crosses* Aluminum chloride with NaCl and KCl *Crossed circles* Do without salts *Triangles* Calcium carbonate without salts *Circles* Calcium chloride without salts *Squares* Magnesium chloride without salts

an ulcer patient), it is concluded that gastric mucosa is digested at a considerable rate throughout the whole pH range of peptic and catheptic activities*) Further the pH values obtained by the most potent inhibiting antacids lie near each other, and the other antacids are poor inhibitors Thus, the third factor is without importance for the present discussion and the order of the antacids for inhibiting the proteolysis of gastric mucosa is the same as shown in tab 3

When the antacids dissolve metal ions are formed (an exception is the group of ion exchange antacids) Therefore the ability of these ions (Al^{+++}, Ca^{++}, and Mg^{++}) to inhibit enzyme activity was also investigated Ca^{++}

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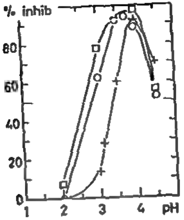


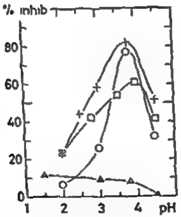


Fig. 9 Abscissa and ordinate as in fig. 8 Substrate 1 6% (w/v) CO hemoglobin *Crosses* Aluminum glycinate *Circles* Dimagnesium aluminum trisilicate *Squares* Aluminum hydroxide, dried gel, F-2200 *Triangles* Bismuth subcarbonate

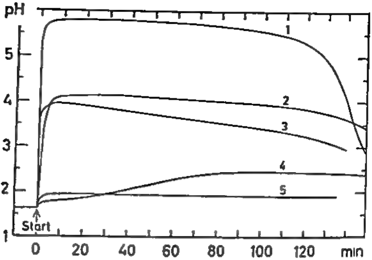
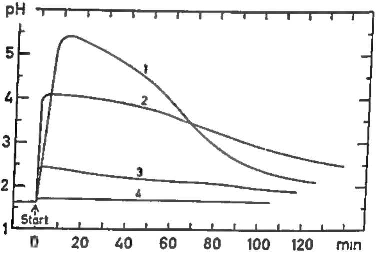


Fig 10 … carbonate … aluminun … Calcium … agnes um … d F 2000



BEEK… … id

and Mg++ did not influence the activity at the concentrations and pH values investigated Al+++, on the other hand, showed inhibiting proper ties at higher pH values (see figs 7 and 8, and tab 4) This applied when the solutions contained 0 1 M NaCl and 0 01 MKCl per litre During the course of this part of the investigation it was discovered (independently of SCHLAMOWITZ & PETERSON 1961) that Na+ and K+ ions stimulate enzyme activity (see fig 3) If the Na+ and K+ ion concentrations were low, Ca++ and Mg++ ions were also stimulators (see tab 4 and fig 7) These results argue in favour of the aluminum containing antacids In aluminum hydroxide-magnesium carbonate, co dried gel, the hydroxide

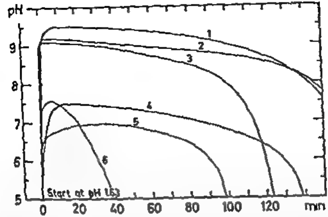


Fig. 12. Dynamic pH time curves established by method of BEEKMAN (1960). 1 Magnesium oxide. 2 Magnesium perhydrol pulver ® magnesium peroxide. 3 Magnesium glycinate. 4 Magnesium subcarbonate. 5 Sodium bicarbonate. 6 Calsil ® calcium silicate.

Table 4

Influence of some inorganic salts on the proteolytic activity of commercial pepsin preparations at pH 1.5 and 3.1. Standard enzyme activity: 0.033 mg tyrosine liberated/ml · min. Substrate: 1.6% (w/v) CO hemoglobin. Temperature: 37°C

Substances	Per cent change of activity with 0.1 M NaCl and 0.01 M KCl	Per cent change of activity without 0.1 M NaCl and 0.01 M KCl	Digestion and pre-inc. pH	Substance conc.
Aluminum chloride	0	0	1.5	30 mM
	−45	−15	3.1	
Calcium chloride	0	+5	1.5	15 mM
	0	+50	3.1	
Magnesium chloride	0	0	1.5	30 mM
	0	+90	3.1	

is in great excess, as the gel contains 40% hydroxide, as Al_2O_3, and 6% carbonate, as MgO.

The dependence of the inhibition on antacid concentrations are shown in figs. 4–7. From these figures it is easily seen that the inhibition did not change proportionately to the antacid concentrations. Relatively the inhibition is higher at a low than at a high concentration. This is a consequence of the fact that the inhibition is an adsorption phenomenon. The pH dependence of the inhibition and the liberation of the enzyme activity after the antacids have been dissolved are also consequences of adsorption.

The co-dried gel did not inhibit the activity of a crystalline trypsin

preparation at pH 5 5 to 7 5 In these experiments the antacid "concentration" was 100 mg/12 ml and the enzyme activity about 0 03 mg tyr lib /ml min

Relevance of obtained results As the inhibition is a consequence of adsorption of the enzyme on the antacid and the adsorbed enzyme is inactive (however, cf above), the *percentage inhibition* ought not to be the same at different concentrations (the initial enzyme activity/antacid "conc " ratio is assumed to be the same) This is easily derived from the adsorption isotherms The following equation is derived from the adsorption isotherm of FREUNDLICH

$$\frac{100\,X}{X + C_x\,V} = \frac{100\,Y}{Y + C_y\,V}\left(\frac{C_x}{C_y}\right)^n,$$

X = amount of adsorbed substance in the first case
Y = amount of adsorbed substance in the second case
C_x = equilibrium conc of X in the solution in the first case
C_y = equilibrium conc of Y in the solution in the second case
V = the volumes of the solutions n = constant, less than one

It is easily shown that C_x is larger than C_y, if $X + C_x V$ is assumed to be larger than $Y + C_y V$, and the initial enzyme activity/antacid 'conc' ratio is the same in both instances Thus the percentage adsorption (inhibition) is *larger* in the more concentrated solution However, no changes in the order of the antacids for degree of inhibition will occur on applying the *in vitro* results to *in vivo* conditions since the *in vitro* and *in vivo* concentrations are close to one another from an adsorption point of view

As shown in fig 1, it took 6 minutes for the adsorption to reach equilibrium under our experimental conditions At higher concentrations of enzyme and antacid, equilibrium will be reached more rapidly This is easily understood if certain facts are taken into account 1) The adsorption rate is proportional to the number of impacts of the enzyme with the antacid surface per time and surface unit, and this number is proportional to the enzyme concentration 2) This concentration is always higher in the more concentrated solutions (*i e* the solutions whose equilibrium concentrations are C_x, see above)

Summary

Several commonly used antacids were examined for their ability to inhibit proteolytic activity in pepsin preparations

The pH of the incubation mixtures was controlled by adding small volumes of acid with the aid of a pH-stat instead of by using strong buffer solutions

The inhibition of proteolysis was shown to be dependent on pH Therefore, the inhibiting abilities of the antacids were compared at pH values expected to occur in gastric juice in the stomach, as judged from model experiments

Inhibition by the ions occurring when the antacids went into solution (Al^{+++}, Mg^{++}, and Ca^{++}) was also investigated Al^{+++} was the only ion inhibiting proteolytic activity

The proteolytic activity of the pepsin preparations was dependent on the Na^{+} and K^{+} concentrations of the solutions

Strongly alkaline antacids were powerful inhibitors of proteolysis However in ulcer therapy it is generally considered more suitable to use "milder" antacids, giving an intragastric pH of about 3 5 In this group of antacids aluminum hydroxide-magnesium carbonate, co-dried gel, was found to be the most potent inhibitor, with aluminum glycinate, dimagnesium aluminum trisilicate, and aluminum hydroxide, dried gel, in order of decreasing activity

Acknowledgements

The authors thank Dr Håkan Westling for valuable discussions and Mr Nils G Nilsson for establishing the dynamic pH-time curves

Added in proof

Hollander stated in a recent paper (Hollander, F Recent advances in the physiology of gastric secretions *Ann N Y Acad Sci* 1962, 99, 4-8) that pure antacid therapy is inadequate for the management of peptic ulcer as the corrosive power of gastric juice is not eliminated merely by increasing pH This statement is in accordance with the opinion of the present authors However, the ways to eliminate the proteolytic activity of gastric juice suggested by *Hollander* (1962) are different from that presented in this paper

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Acta pharmacol et toxicol 1962, 19, 235–240

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The in vivo Transformation of Morphine and Nalorphine into Normorphine in the Brain of Rats

By

Kirsten Milthers

(Received June 20, 1962)

There are important pharmacologic implications (WAY & ADLER 1960) for the N de alkylation of analgesics According to the hypothesis of BECKETT, CASY & HARPER (1956) the nor derivative is responsible for the analgesic effect, whereas AXELROD (1956 b) attributes the N-demethylation to the development of tolerance

However, the N-demethylation of morphine *in vivo* has not been confirmed until recently (MISRA, MULÉ & WOODS 1961; MILTHERS 1962) and the demonstration of the presence of *nor*morphine in the brain has not so far been accomplished

Using a method recently described (MILTHERS 1961) we have shown quantitatively the presence of *nor*morphine in the brain of normal and hepatectomised rats after the administration of either morphine or *nalorphine* Further, our experiments suggest that the transformation of morphine into *nor*morphine occurs in the brain, and not only in the liver as shown by AXELROD (1956 a) in experiments in vitro

The results of some of these experiments have already been briefly described (MILTHERS 1962)

Methods

The *substances used* were *nor*morphine chloride ($C_{16}H_{17}O_3N \cdot HCl\ H_2O$), (M P 305°–307°), obtained from the Department of Health, Education and Welfare, Washington D C U S A, *nalorphine* chloride ($C_{19}H_{21}O_3N\ HCl$) (Anarcon ®) [illegible]EA Pharmaceutical Products Copenhagen Denmark [illegible]

mental conditions with free access to water and a commercial food preparation up to the experimental period. The rats were anaesthetized with mebumal sodium (pentobarbital sodium), 40 mg/kg given intraperitoneally dissolved in 5 mg/ml saline. During the experimental period oxygen was given through a tracheal tube. An aqueous solution of either morphine or *nalorphine* (40 mg/ml) was injected intravenously into the brachial vein in amounts of 100 mg/kg. The rats were killed 15 minutes later by decapitation. For the determinations of morphine, *nalorphine* and *normorphine*, samples of blood were taken from the severed neck. Then the whole brain, including the brain stem and cerebellum, was removed.

Hepatectomy. To prevent stasis it was necessary to remove the intestines, pancreas and spleen as well as the liver. Before resection ligatures were placed on the rectum, the cardia, the mesenterial radix and on each lobe of the liver separately. Care was taken not to interfere with the vena cava at the dorsal side of the liver. In this way more than 95% of the liver could be succesfully removed.

Determination of morphine, nalorphine, and normorphine. The concentrations of the substances in the blood and brain were determined as described by MILTHERS (1961). The average recoveries of morphine, *nalorphine* and *normorphine* were 96% 96% and 72%, respectively. When calculating the concentrations of *normorphine* allowance was made for the low percentage recovery, and the values were converted to morphine or *nalorphine*.

Saline only was injected into the brachial vein of two hepatectomised rats. The rats were killed 15 minutes later, and the blood and brain were examined for morphine, *nalorphine*, and *normorphine*. None of these samples gave positive result.

Table 1

Concentrations of total (free and conjugated) *morphine and normorphine* in the blood and brains of normal and hepatectomised rats 15 min after intravenous injection of 100 mg/kg *morphine*

Normal rats					Hepatectomised rats				
Weight g	Conc. in the blood µg/ml		Conc. in the brain µg/g		Weight g	Conc. in the blood µg/ml		Conc. in the brain µg/g	
	Morphine	*Normorphine*	Morphine	*Normorphine*		Morphine	*Normorphine*	Morphine	*Normorphine*
270	74	3.1	6.8	<0.7	250	105	1.5	23	1.0
270	66		10.9		270			21	
285	60	5.0	7.1	<0.7	290			24	1.6
280	47		7.2		250			22	
310	64[1]	3.6[2]	6.?[3]	0.4	270			28	1.7
295					280			27	
315	59[1]				250			34	1.0
315					290			28	
Mean	62	3.8	7.1					26	1.3

1) ... → 36 µg of the total 59 µg
2)
3)

Results

The concentrations of morphine and *nor*morphine in the blood and brain from normal and hepatectomised rats 15 minutes after intravenous injection of 100 mg/kg morphine are given in table 1

The figures show the total amounts of conjugated and unconjugated ('free') compound The scarcity of the material available did not permit determination of both free and conjugated compound However, blood from some of the normal rats was pooled, and in this 50–60% of the total morphine was found as free morphine, whereas 60–70% of the total *nor*morphine was demonstrated as free *nor*morphine Only the free compound was found in the brain

In the brain of normal rats given morphine (100 mg/kg) *normorphine* could not be demonstrated by the method of determination referred to above The concentration of morphine found in the brain of these rats was only 7 1 µg/g The average concentration of morphine and *nor*morphine in the blood were 62 µg/ml and 3 8 µg/ml, respectively

In hepatectomised rats the concentrations of morphine in the blood and the brain were 105 µg/ml and 26 µg/g and of *nor*morphine 1 5 µg 1 ml and 1 3 µg/g, respectively

The concentrations of *nal*orphine and *nor*morphine in the brains of normal or hepatectomised rats 15 minutes after intravenous injection of 100 mg/kg *nal*orphine are given in Table 2 In normal rats we found 28 µg/g *nal*orphine and 1 2 µg/g *nor*morphine In hepatectomised rats the concentration of *nal*orphine and *nor*morphine were 278 µg/g and 4 9 µg/g respectively

Table 2

Concentrations of total (free and conjugated) *nalorphine and normorphine* in the brains of normal and hepatectomised rats 15 min. after intravenous injection of 100 mg/kg *nalorphine*

Normal rats			Hepatectomised rats		
Weight g	Conc in the brain µg/g		Weight g	Conc in the brain µg/g	
	Nalorphine	Normorphine		Nalorphine	Normorphine
215	18	1 1	260	230	7 0
215	29		280	239	
210	35	1 3	280	272	2 7
215	28		290	285	2 9
Mean	28	1 2		257	4 9

mental conditions with free access to water and a commercial food preparation up to the experimental period The rats were anaesthetized with mebumal sodium (pentobarbital sodium), 40 mg/kg given intraperitoneally dissolved in 5 mg/ml saline During the experimental period oxygen was given through a tracheal tube An aqueous solution of either morphine or nalorphine (40 mg/ml) was injected intravenously into the brachial vein in amounts of 100 mg/kg The rats were killed 15 minutes later by decapitation For the determinations of morphine, nalorphine and normorphine, samples of blood were taken from the severed neck Then the whole brain, including the brain stem and cerebellum, was removed

Hepatectomy To prevent stasis it was necessary to remove the intestines pancreas and spleen as well as the liver Before resection ligatures were placed on the rectum, the cardia the mesenterial radix and on each lobe of the liver separately Care was taken not to interfere with the vena cava at the dorsal side of the liver In this way more than 95% of the liver could be succesfully removed

Determination of morphine, nalorphine, and normorphine The concentrations of the substances in the blood and brain were determined as described by MILTHERS (1961) The average recoveries of morphine, nalorphine and normorphine were 96% 96% and 72%, respectively When calculating the concentrations of normorphine allowance was made for the low percentage recovery, and the values were converted to morphine or nalorphine

Saline only was injected into the brachial vein of two hepatectomised rats The rats were killed 15 minutes later, and the blood and brain were examined for morphine nalorphine, and normorphine None of these samples gave positive result

Table 1

Concentrations of total (free and conjugated) *morphine and normorphine* in the blood and brains of normal and hepatectomised rats 15 min after intravenous injection of 100 mg/kg *morphine*

Normal rats					Hepatectomised rats				
Weight g	Conc in the blood µg/ml		Conc in the brain µg/g		Weight g	Conc in the blood µg/ml		Conc in the brain µg/g	
	Morphine	*Normorphine*	Morphine	*Normorphine*		Morphine	*Normorphine*	Morphine	*Normorphine*
270	74	3 1	6 8	<0 7	250	*105*	*1 5*	23	1 0
270	66		10 9		270			21	
285	60	5 0	7 1	<0 7	290			24	1 6
280	47		7 2		250			22	
310	64[1]	3 6[2]	6 2[3]	0 4	270			28	1 7
295					280			27	
315	59[1]				250			34	1 0
315					290			28	
Mean	6[illegible]	3 8	7 1					26	1 3

1) Free morphine amounted to 33 µg of the total 64 µg and to 36 µg of the total 59 µg
2) Free *normorphine amounted to 2 4 µg of the total 3 6 µg*
3) All the morphine was found as free morphine

only instance where the phenomenon of de-alkylation is known to occur (i.e. by the liver enzymes). Our results indicate that the de-alkylation of morphine occurs more readily *in the brain* than that of *nal*orphine, since the *nor*morphine found in the brain of hepatectomised rats amounts to more than 5% of the morphine and to less than 2% of the *nal*orphine. However, this observation does not change the fact that the absolute amount of *nor*morphine is found to be higher in the brain after administration of *nal*orphine because of the correspondingly higher amount of nalorphine. Nevertheless, the higher amount of *nor*morphine found in the brain after administration of *nal*orphine is also inconsistent with the hypothesis of Beckett because *nal*orphine has a lower analgesic activity than morphine.

Summary

The concentrations of morphine or *nal*orphine and of *nor*morphine in the brain and blood of normal and hepatectomised rats after intravenous administration of either 100 mg/kg morphine or *nal*orphine have been studied.

1. *Nor*morphine could be demonstrated in the brain of rats after intravenous injection of either morphine or *nal*orphine.

2. Evidence has been obtained for the in vivo transformation of both morphine and *nal*orphine into *nor*morphine *in the brain* of rats.

3. N-de-alkylation of morphine occurs more readily than that of *nal*orphine in the brain of rats.

Acknowledgements

Nalorphine was kindly supplied by J. Gad Andresen (deceased 26/8 1961). I am sincerely grateful to P. V. Petersen of H. Lundbeck & Co. Ltd., Copenhagen, for forwarding a sample of normorphine supplied by the Department of Health, Education and Welfare, Washington D.C., U.S.A.

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Discussion

The concentrations of morphine and *nal*orphine in the brains of hepatectomised rats were considerably higher than those of the normal rats This is understandable, since the conjugation of the substance occurs mainly in the liver and only the free compounds pass the blood brain barrier The difference in concentration of *nal*orphine in the brains of normal and hepatectomised rats is exceptionally marked Probably this is due to the fact that *nal*orphine is conjugated to a greater extent (WOODS & MUEHLENBECK 1957)

The higher concentration of morphine in the blood of hepatectomised rats than in that of normal rats may be explained by the reduction in body weight produced by evisceration

AXELROD (1956 a) has demonstrated the transformation of morphine into *nor*morphine *in vitro* by microsomes prepared from the livers of male rats, but microsomes from kidney, brain, muscle or spleen showed no such activity The results of our investigations indicate that morphine and *nal*orphine are transformed into *nor*morphine *in the brain of living rats* The ratio of the blood/brain distribution of free morphine is about the same in normal rats (34 5/6 2 (average of the four lower rats)) as in hepatectomised rats (105/22 5 (average of the four upper rats)) after intravenous administration of morphine In the hepatectomised rats the substance would have to be mainly unconjugated Thus, hepatectomy does not seem to influence the passage of morphine through the blood brain barrier and it therefore appears unlikely that it could affect the passage of *nor*morphine Nevertheless, the blood/brain distribution ratio of free *nor*morphine is only about 1 (1 5/1 3) in hepatectomised rats, whereas the ratio is greater than 6 (>2 4/0 4) in normal rats The concentration of *nor*morphine *in the blood* from the hepatectomised rats is small in spite of the high concentration of morphine, probably because the main site of action of the N demethylation is removed At the same time the concentration of *nor*morphine *in the brain* of these animals is relatively high Thus, most of the *nor*morphine appears to be transformed from morphine *in the brain itself*, since only a very small part may have passed the blood brain barrier Indeed, this observation supports the much debated hypothesis of BECKETT *et al* (1956) though this is still far from having been proved

LASAGNA & KORNFELD (1958) have stressed that this hypothesis only could be reconciled with the relatively low potency of *nor*morphine if *nor*morphine is formed in cell receptor sites after the entry of morphine into them In their comprehensive discussion of this hypothesis WAY & ADLER (1960) have pointed out as evidence to the contrary that de alkylation of *nal*orphine occurs more readily than does that of morphine in the

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Morphine and Normorphine in the Brain of Rats A Comparison of Subcutaneous, Intraperitoneal, and Intravenous Administration

By

Torkell Johannesson and Kirsten Milthers

(Received June 20 1962)

It has recently been shown that the N-demethylation of morphine into normorphine may occur in the brain of rats (MILTHERS 1962) This observation may be of outstanding importance for the hypothesis of BECKETT CASY & HARPER (1956) which assumes morphine to be N demethylated before exerting its analgesic action One of the predictions from this hypothesis is that morphine and normorphine are drugs of almost equal analgesic potency However, several authors have found normorphine to be less active than morphine as an analgesic (MILLER & ANDERSON 1954, LASAGNA & DE KORNFELD 1958, HORLINGTON & LOCKETT 1959)

The weaker action of normorphine has been explained as possibly due to its more rapid destruction in the body and possibly less efficient permeation into the brain (LOCKETT & DAVIS 1958), but the validity of these explanations has not been tested previously In the investigation reported below we have therefore compared the concentrations of morphine and normorphine in the brain of rats The above mentioned drugs were injected subcutaneously intraperitoneally or intravenously

Methods

Adult male albino rats were used The rats were kept in constant environmental conditions with free access to water and a commercial food preparation Experiments were conducted at room temperature (approximately 22 C) Respiratory arrest was considered a sign of death

Administration of drugs Morphine chloride (Ph Dan 1948) or normorphine chloride (obtained from The Department of Health Education and Welfare, Washington D C., U.S.A) was given in aqueous solutions containing 40 mg/ml for subcu-

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Table 2

Concentrations of morphine and normorphine in the brains of rats after intraperitoneal injection of 500 mg/kg of the drug All the rats given morphine were killed whereas all the rats given normorphine died

Rats injected with morphine			Rats injected with normorphine		
Weight g	Time after inj minutes	Conc of morphine in the brain µg/g	Weight g	Time after inj minutes	Conc of normorphine in the brain µg/g
145	15	11	140	16	12 0
145	15	12	160	14	9 5
145	20	14	135	15	7 9
150	20	15	145	23	8 2
190	18	15	160	15	14 0
185	18	12	170	17	7 2
190	20	10	155	24	11 0
200	20	21	195	21	8 5
Mean	18	14		18	9 8

in table 1 Although the concentrations of morphine were low, it could be demonstrated in 6 out of 10 rats The measurable amounts ranged from 1 4 to 4 3 µg/g brain Normorphine was found in amounts even lower than those of morphine, it could only be measured in 2 out of 10 rats In these two rats the concentrations were 1 µg/g and 2 µg/g brain

Intraperitoneal Administration

Morphine or normorphine was administered intraperitoneally to the rats in amounts of 500 mg/kg All the rats that received normorphine died 15–25 minutes (average, 18 minutes) after the injection For comparison purposes the rats that received morphine were killed at approximately the same times The concentrations of the drugs in the brain are given in table 2 On the average the morphine concentration was 14 µg/g brain, whereas normorphine was found present at a clearly lower concentration, averaging 9 8 µg/g brain

Intravenous Administration

Morphine or normorphine was administered intravenously to the rats in amounts of 50 mg/kg They were killed 15 minutes after the injection

The concentrations of morphine and normorphine in the brain are given in table 3 The average concentration of morphine was 3 4 µg/g brain The concentrations of normorphine were the same as those of morphine, the average was 3 6 µg/g brain

taneous and intraperitoneal injections Subcutaneous injections were given in the midline 1–2 cm above the root of the tail, and intraperitoneal injections were given in the upper part and the right side of the abdomen

For intravenous injections solutions of 8 mg/ml were made in distilled water The injections were given into the right brachial vein over periods of two minutes The rats receiving intravenous injections were anaesthetized previously with mebumal sodium (pentobarbital sodium) 40 mg/kg, injected intraperitoneally During the experimental period (15 minutes) oxygen was given through a tracheal tube

Determinations of morphine and normorphine in the brain (including brain stem and cerebellum) were performed by the method of MILTHERS (1961) The average recoveries were about 96% and 72% for morphine and normorphine, respectively The lowest measurable amount is about 1 µg/g brain In calculating the concentrations of normorphine allowance was made for the low percentage recovery

In order to exclude any interference from the anaesthesia on the determination of morphine and normorphine, saline was injected into the brachial vein of two rats that had received the usual anaesthesia and oxygen Fifteen minutes later the rats were decapitated and their brains analysed for morphine and normorphine None of the samples gave positive results

Results

Subcutaneous Administration

Morphine or normorphine was administered subcutaneously to rats in amounts of 200 mg/kg The rats were little affected by these doses and were killed 15 minutes after being injected

The concentrations of morphine or normorphine in the brain are given

Table 1

Concentrations of morphine and normorphine in the brains of rats 15 minutes after subcutaneous injection of 200 mg/kg of the drug

Rats injected with *morphine*		Rats injected with *normorphine*	
Weight g	Conc of morphine in the brain µg/g	Weight g	Conc of normorphine in the brain µg/g
150	1 4	155	n d a
150	4 3	155	trace
150	trace	160	2 0
150	trace	160	1 0
150	1 6	160	n d a
150	1 9	160	n d a
150	2 1	160	trace
150	2 2	150	n d a
150	n d a	150	n d a
150	trace	150	trace

n d a , no demonstrable amount s
trace amounts not permitting quantitative determination

Table 2

Concentrations of morphine and normorphine in the brains of rats after intraperitoneal injection of 500 mg/kg of the drug All the rats given morphine were killed, whereas all the rats given normorphine died

Rats injected with morphine			Rats injected with normorphine		
Weight g	Time after inj minutes	Conc of morphine in the brain µg/g	Weight g	Time after inj minutes	Conc of normorphine in the brain µg/g
145	15	11	140	16	12 0
145	15	12	160	14	9 5
145	20	14	135	15	7 9
150	20	15	145	23	8 2
190	18	15	160	15	14 0
185	[illegible]	12	170	17	7 2
190	20	10	155	24	11 0
200	20	21	195	21	8 5
Mean	18	14		18	9 8

in table 1 Although the concentrations of morphine were low, it could be demonstrated in 6 out of 10 rats The measurable amounts ranged from 1 4 to 4 3 µg/g brain Normorphine was found in amounts even lower than those of morphine, it could only be measured in 2 out of 10 rats In these two rats the concentrations were 1 µg/g and 2 µg/g brain

Intraperitoneal Administration

Morphine or normorphine was administered intraperitoneally to the rats in amounts of 500 mg/kg All the rats that received normorphine died 15–25 minutes (average, 18 minutes) after the injection For comparison purposes the rats that received morphine were killed at approximately the same times The concentrations of the drugs in the brain are given in table 2 On the average the morphine concentration was 14 µg/g brain, whereas normorphine was found present at a clearly lower concentration, averaging 9 8 µg/g brain

Intravenous Administration

Morphine or normorphine was administered intravenously to the rats in amounts of 50 mg/kg They were killed 15 minutes after the injection The concentrations of morphine and normorphine in the brain are given in table 3 The average concentration of morphine was 3 4 µg/g brain The concentrations of normorphine were the same as those of morphine, the average was 3 6 µg/g brain

Table 3

Concentrations of morphine and normorphine in the brains of rats 15 minutes after intravenous injection of 50 mg/kg of the drug

Rats injected with morphine		Rats injected with normorphine	
Weight g	Conc of morphine in the brain µg/g	Weight g	Conc of normorphine in the brain µg/g
125	3 1	110	2 8
115	3 0	125	3 9
130	2 9	120	2 8
120	3 3	120	3 2
110	3 3	125	3 2
130	3 7	120	3 8
130	3 1	125	3 3
135	3 8	125	3 6
130	4 1	105	5 7
145	3 4		
Mean	3 4		3 6

Discussion

On the average morphine was found at a higher concentration than normorphine in the brain of rats when the drugs were given subcutaneously and intraperitoneally, whereas the concentrations of both drugs were the same when they had been given intravenously

The results indicate that the small quantities of normorphine in the brain, as compared with morphine, may be caused by a slower rate of absorption, rather than by more rapid destruction in the body or by slower passage from the blood into the brain, as suggested by LOCKETT & DAVIS (1958) A plausible explanation of this occurrence could be a more marked liberation of histamine and enteramine caused by normorphine MILTHERS & SCHOU (1958) and MILTHERS (1959) have shown self-depression in subcutaneous absorption of morphine due to liberation of histamine and enteramine (5-hydroxytryptamine) The self-depression of absorption of *normorphine is possibly more pronounced than that of morphine*, but the results of experiments confirming this assumption are not available at present

Several authors have studied the analgesic potency of normorphine compared with that of morphine On *subcutaneous administration* to man (LASAGNA & DE KORNFELD 1958), dogs and rats (ORAHOVATS & LEHMAN 1958) and mice (MILLER & ANDERSON 1954), normorphine was found to be a less potent analgesic than morphine The potency ratios (normorphine/morphine) ranged from 1/2 to 1/18 By *intraperitoneal administration* to mice (MILLER & ANDERSON 1954) similar results were obtained By

intravenous administration to dogs, ORAHOVATS & LEHMAN (1958) found the analgesic potency of normorphine equal to that of morphine, whereas LOCKETT & DAVIS (1958) in experiments with mice found normorphine to be less active than morphine

In our experiments with rats, we used large amounts of morphine and normorphine However, according to our own unpublished results there seems to be approximately the same relation between the concentrations of the two drugs in the brains of rats that received intraperitoneal injections of 50 mg/kg or 500 mg/kg Further the drugs were found in the same concentrations in the brain of rats whether 50 mg/kg normorphine or 20 mg/kg morphine were given intraperitoneally Thus it appears that morphine ought to be found in higher amounts than normorphine if the drugs are given in appropriate analgesic doses by the subcutaneous and the intraperitoneal route, whereas no difference would be expected by the intravenous route

Our results, compared with the analgesic investigations mentioned above, are in full accordance with the view 1) that the lower analgesic effect of normorphine, given subcutaneously or intraperitoneally, is conditioned by lower amounts in the brain and 2) that morphine and normorphine are drugs of almost equal analgesic potency, as was suggested by BECKETT, CASY & HARPER (1956)

Summary

1 Morphine and normorphine have been administered to rats by subcutaneous, intraperitoneal and intravenous injection

2 Morphine was found in the brain in higher amounts than normorphine when the subcutaneous and intraperitoneal route had been used When given intravenously, both drugs were found at the same concentrations in the brain

3 The lower concentration of normorphine than of morphine in the brain may be explained by a greater self-depression of the absorption of normorphine

4 The view that morphine and normorphine are drugs of equal analgesic potency is supported by the experimental results

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Anticonvulsant Action of some New Skeletal Muscle Relaxants on Strychnine Convulsions in Mice

By

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(Received June 23, 1961)

In recent years numerous effective skeletal muscle relaxants have been introduced These substances selectively inhibit hyperactive polysynaptic reflexes in the spinal cord and subcortical centres (LICHTMAN 1958; O'DELL 1960, RANDALL 1961), but they have no direct effect on muscles or peripheral nerves (LICHTMAN 1958) It has been claimed that all these agents have anticonvulsive properties Our investigation was intended to compare the anticonvulsant properties of some new skeletal muscle relaxants with that of phenobarbital in strychnine convulsions

Material and Methods

White male mice weighing from 20 to 25 g served as test animals The convulsions were induced by 1 mg/kg of strychn[illegible] cor[illegible] no[illegible] So[illegible] dia[illegible] (Librium ®, F Hoffmann La Roche & Co A/G*), phenyramidol (Analexin ®, Oy Orion, Helsinki*), methocarbamol (Robaxin ®, A H Robins & Co*) and sodium phenobarbital They were injected intraperitoneally at the same time as the strychnine

Phenyramidol, chlordiazepoxide and sodium phenobarbital were used in aqueous solution Chlorzoxazone was dissolved in 1% NaOH solution, mephenesin and methocarbamol in 10% polyethylenglycol solution and chlormezanone in a solution containing 4% ethanol and 40% propylene glycol The solvents mentioned above were tested separately in greater volumes than those used in our investigation No convulsions or other effects were observed and the effect of strychnine was not changed by any of them.

The doses given refer to the corresponding base or acid

The LD50 of each agent was measured, the observation time being limited to four

*) We are grateful to these companies for the gift of substances used

hours The anticonvulsant activity was evaluated by establishing the dose that prevented spontaneous strychnine convulsions (CPD 50) and the dose that prevented death induced by strychnine (DPD50) For each of these three values we used from 35 to 60 test animals at a room temperature about 24° After the injections the animals were kept separately in glass jars with as little disturbance from outside as possible

Calculation of the values and their standard errors was done by the method of MILLER & TAINTER (1944) on semilogarithmic millimetre paper

Results

The results are shown in table I The ratios of LD50 to death preventing dose 50 (LD50/DPD50) and that of LD50 to convulsion preventing dose (LD50/CPD50) are also shown in the table

Table I

LD 50 dose preventing strychnine convulsions (CPD 50), dose preventing strychnine death (DPD 50), the ratios LD 50 DPD 50 and LD 50/CPD 50 of the examined substances

Substance	LD 50 mg/kg	DPD 50 mg/kg	CPD 50 mg/kg	LD 50/ DPD 50	LD 50/ CPD 50
Chlordiazepoxide	392 ± 13.3	9.4 ± 1.9	34.0 ± 2.5	41.7	11.5
Chlormezanone	322 ± 11.0	11.5 ± 1.3	20.5 ± 2.5	35.8	15.7
Chlorzoxazone	170 ± 6.7	44 ± 3.2	68 ± 5.5	3.9	2.4
Mephenesin	285 ± 10.1	57 ± 8.2	148 ± 6.9	5.0	1.9
Methocarbamol	780 ± 34	175 ± 4.6	230 ± 17.1	4.5	3.4
Phenobarbital	248 ± 16.7	26.5 ± 4.0	82 ± 9.5	9.4	3.0
Phenyramidol	405 ± 9.5	83 ± 6.3	–	4.5	–

The ability of chlordiazepoxide and chlormezanone to prevent both convulsions and death induced by strychnine was unequivocally higher than that of the other substances tested There was no obvious difference between the actions of chlorzoxazone, mephenesin, methocarbamol and phenobarbital Although it prevented death, phenyramidol could not inhibit the convulsions even when administered in doses as high as the LD50

Discussion.

Phenobarbital was used in this study because it has a known value in strychnine convulsions (HAGGARD & GREENBERG 1932, KEMPF, MCCALLUM & ZIRFAS 1933) In our experiments only chlordiazepoxide and chlormezanone were superior to phenobarbital in preventing the death induced by strychnine For preventing convulsions phenobarbital was about as effective as chlorzoxazone, mephenesin and methocarbamol

Here again chlordiazepoxide and chlormezanone were clearly superior to the others

In previous experiments (RANDALL 1961) on strychnine convulsions in mice, the ratio LD50/DPD50 was reported to be 5 3 for phenobarbital and 8 3 for chlordiazepoxide, compared with 9 4 and 41 7 in our experiments In the tests quoted above, however, the strychnine dose was 2 mg/kg instead of 1 mg/kg, as used by us

ROSZKOWSKI (1960) has determined LD50, DPD50 and CPD50 for several agents, including chlorzoxazone, mephenesin and methocarbamol If the relations LD50/CPD50 and LD50/DPD50 are calculated, the values obtained are chlorzoxazone 0 9–1 3, mephenesin 1 3–1 9 and methocarbamol 1 9–3 4 The dose of strychnine was 1 5 mg/kg These ratios are somewhat lower than ours Further, in our experiments the anticonvulsant property of methocarbamol was not obviously greater than that of the two other compounds

We have not found any detailed information about the anti strychnine properties of chlormezanone and phenyramidol, whereas the anti strychnine properties of chlormezanone have been reported (GESLER & SURREY 1958) The anticonvulsant properties of phenyramidol have been regarded as almost non-existent (O'DELL *et al* 1960) Our results point to the same conclusion

Summary

The anticonvulsant effects of phenobarbital, chlodiazepoxide, chlormezanone chlorzoxazone, mephenesin methocarbamol and phenyramidol in strychnine convulsions in mice have been investigated The LD50, the dose preventing convulsions (CPD50) and the dose preventing death (DPD50), were recorded In order to be able to compare the activities of the various substances the ratios LD50/DPD50, and LD50/CPD50 were calculated

Chlordiazepoxide and chlormezanone were superior to the other substances tested Chlordiazepoxide had the highest LD50/DPD50 and chlormezanone the highest LD50/CPD50 Phenyramidol could not prevent convulsions at any dose level The activities of the other substances investigated were almost identical

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Influence of some Antirheumatic Compounds on Formation of Granulation Tissue

By

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(Received June 29, 1962)

It has long been recognised that glucocorticoids inhibit the healing of wounds However, in therapeutic doses to man these hormones seem to have no deleterious effect on the processes of healing (HALEY & WILLIAMSON 1962) It is different in animal experiments, in which large hormone doses are used The glucocorticoids are known to have considerable anti-inflammatory action, and certain experimentally established facts suggest that an initial inflammatory reaction is of considerable importance for initiating regenerative processes The object of our study has been to establish whether the glucocorticoids may be considered to act on the formation of granulation tissue *via* inhibition of the traumatically determined inflammatory reaction Further, we have aimed at throwing some light on the question whether the glucocorticoids have an activity pattern similar to that of other anti inflammatorily acting drugs, such as phenyl butazone and sodium salicylate

Methods

Female rats weighing on average 100 g were used

Schedules of Treatment

All the drugs mentioned below were dissolved in 0 5 ml of physiological saline and injected daily subcutaneously into the occipital region The daily doses were, for *phenylbutazone* 80 mg/kg for *sodium salicylate* 200 mg/kg for *hydrocortisone* 12 5 mg/kg and for *prednisone* 2.5–5 0 or 10 mg/kg The controls were given injections of physiological saline in the same way as the treated animals

The drug treatment was begun 5 days before surgical intervention and continued until the animals were killed.

Formation of granulation tissue The technique described by RUDAS (1960) was employed With the rat under ether anaesthesia, a circular piece of skin was excised medially and distally on the back A plastic ring was inserted in the resultant hole The

diameter of the ring was so much larger than the hole that the ring was kept fixed there Further, the ring was provided with flanges to prevent its slipping off All the rings had exactly the same inner diameter (20 mm), so that quantitative measurements of the granulation tissue formed were possible The animals were killed 8 days later and the rings were removed The granulation tissue formed was then easily separated by blunt dissection from the underlying muscular fascia The tissue was weighed on a torsion balance, immediately after excision and then frozen at −25°C After freezing the tissue was dried in a desiccator over silica gel at room temperature and a pressure of 1–3 mm Hg It was then reweighed on the torsion balance This procedure was repeated Defatting was performed in special perforated metal capsules by shaking twice in a bath containing light petroleum for 30 minutes, with several subsequent shakings in ordinary ether until the weight remained constant Finally after drying in a desiccator, the tissue was weighed on the torsion balance

Collagen determination In granulation tissue the collagen content was determined as hydroxyproline multiplied by the factor 7 46 The dried defatted tissue was hydrolysed with 6 N-HCl for 24 hours at 100° in sealed glass ampoules The hydrolysate was filtered, and the amount of hydroxyproline was measured by MARTIN & AXELROD's (1955) modification of NEUMANN & LOGAN's method

Tensile strength After shaving the coat with an electric clipper a longitudinal incision was made along the middle of the back through the skin and subcutaneous tissue down to the muscular fascia The wound was ligated with silk knots The tensile strength was measured 8 days later Together with the accompanying skin 3 cm of the wound were excised On both sides of the wound the skin was fixed by means of clamps, which were connected to threads One thread was kept fixed, the other connected *via* a pulley to a freely suspended plastic bag The bag was filled with water from a burette at a constant flow rate until the wound burst The amount of water that had flown into the plastic bag by the time the wound burst was used as a measure of the strength of the wound

Results

Table 1 shows the amounts of wet and dry, defatted granulation tissue in milligrams The water content is given as relative water content, i e grams water per 100 g dry, defatted tissue The weight of the wet tissue was reduced in all the treated groups except in the one treated with prednisone doses of 2 5 mg/kg Exactly the same was true of the relative water contents, whereas significant reductions in the amounts of dry defatted tissue were only seen in the two groups treated with prednisone at doses of 5 0 and 10 0 mg/kg It is seen from table 1 that the various control groups differed considerably in the amount of tissue produced This was probably due to the various main groups having been treated at different times of the year The results for each main group of animals treated simultaneously have therefore been enclosed within thick lines in the tables

The hydroxyproline and collagen contents are given in table 2, hydroxyproline as milligrams hydroxyproline per 100 g dry, defatted tissue There was no significant difference in the percentage collagen contents between

Table 1.

The weight of the wet and of dry, defatted tissue and relative water contents of the granulation tissues The weight of the granulation tissues are given in mg the water contents as grammes water per 100 g dry, defatted tissue (relative water content)

	n	Weight of wet tissue		Weight of dry defatted tissue		Water	
		mg	s e m	mg	s e m	g/100 g	s e m.
Control	15	452	16	71	3	532	14
Salicylate	14	363³)	16	62	3	479²)	8
Phenylbutazone	15	366²)	24	66	4	447¹)	33
Hydrocortisone	15	353²)	31	63	5	448¹)	10
Control	22	393	13	54	[illegible]	628	12
Prednisone 2 5 mg/kg	18	373	31	54	4	585	17
Prednisone 5 0 mg kg	20	281¹)	28	44³)	3	509	19
Control	20	451	16	64	2	598	8
Prednisone 10 mg kg	19	305¹)	13	50¹)	3	506	27

¹) Significantly different from control ($P < 0\ 001$)
²) Significantly different from control ($P < 0\ 01$)
³) Significantly different from control ($P < 0\ 02$)

Table 2

Hyd

animals n

	n	Hydroxy proline mg/100 g	Collagen	
			g/100 g	s e m
Control	15	2165	16 15	0 80
Salicylate	14	2030	15 14	0 47
Phenylbutazone	15	2012	15 01	0 75
Hydrocortisone	15	2074	15 47	0 64
Control	22	2055	15 33	0 28
Prednisone 2 5 mg kg)	[illegible]	1886	14 07	0 44
Prednisone 5 mg kg	20	1936	14 45	0 39
Control	20	1989	14 84	0 48
Prednisone 10 mg kg	19	1809	13 50	0 60

Table 3

Tensile strength of linear wounds, not significantly decreased in the treated groups Number of animals in brackets

	Hydrocortisone (11)	Salicylate (14)	Phenylbutazone (13)	Control (16)
g	298 ± 25	321 ± 18	323 ± 34	384 ± 28

any of the treated groups and the control groups, even though the numerical values were lower throughout in the treated groups In the two groups with a significantly reduced production of dry tissue, the total amount of collagen developed was naturally reduced

Table 3 records the tensile strength of wounds in animals given hydrocortisone, phenylbutazone or sodium salicylate in the same doses as the animals treated with the drugs used in the granulation tissue group No significant difference was noticed between the treated animal groups and the control group The strength of the wound is given as grams of water

Discussion

RUDAS (1960), using a technique exactly similar to that employed in our work, found that hydrocortisone, salicylate or phenylbutazone has an inhibiting effect on the production of granulation tissue RUDAS measured only wet weight CAUWENBERGE & LECOMTE (1957 a, b) arrived at a similar result after subcutaneous insertion of cotton plugs into rats and treatment with salicylate, cortisone or phenylbutazone These workers also did not determine the water contents of the tissues

As cortisone can check the development of inflammatory oedema (see, for instance, COOPER, SCHMIDT & SCHOU 1957), and as large doses of cortisone can reduce the water content of the skin of mice (HVIDBERG & SCHMIDT 1959), the idea naturally suggested itself that the recorded fall in the weight of fresh granulation tissue might be caused in part by a reduced amount of water in these tissues This hypothesis may also be said to harmonise with the reduction found histologically in several studies on wound exudate in cortisone-treated animals (SPAIN, MOLOMUT & HABER 1950, LATTES *et al* 1953) As seen in table 1, all the drugs investigated had a pronounced inhibitory effect on the production of wet granulation tissue This effect depends, in fact, in the first place on a reduction in the relative water content A high dosage of prednisone, however, also led to a significant reduction in the amount of dry, defatted granulation tissue

LATTES *et al* (1956) suggest that administration of cortisone may give rise to blocking of a physiological factor produced early in the period of healing and influencing the course of the healing They are of the opinion that this factor is present in tissue rich in chondroitin sulphate To substantiate their theory they have injected suspensions of cartilaginous tissue, powdered bone and tissue from umbilical cord into granulation tissue and thus managed to abolish the depressing action of cortisone on healing Possibly, however, such treatment may only unspecifically provoke a foreign body reaction that cortisone is unable to abolish Experiments similar in principle have been conducted by LINDNER & RUDAS (1961) who sprinkled powdered peptone on open granulation tissue In this way they abolished the inhibitory action seen on single injection of a fairly large dose of phenylbutazone In the same paper they also showed that single doses of sodium salicylate and phenylbutazone greatly inhibited production of granulation tissue, when the drugs were given simultaneously with the formation of the wound, but that the inhibitory action was negligible if the single injection was given a few days later Hydrocortisone had no effect when injected in a single dose at the beginning of healing In their opinion, this is explained by the fact that hydrocortisone at the large dose employed causes the organism to take compensatory measures tending towards abolishing the action of the hormone The possibility also exists however, that hydrocortisone is not absorbed at a sufficiently fast rate to exert its presumed anti inflammatory action The view that the drugs with anti inflammatory action are most active at an early stage of healing is borne out in the work of SAVLOV & DUNPHY (1954) and DUNPHY (1960), where it is stated that the inhibitory action of the glucocorticoids on the healing presupposes pretreatment with the drugs for some time before healing begins

The results of the studies mentioned above are easily interpreted as showing that the anti inflammatory drugs inhibit the regenerative processes – at least to a great extent – by suppressing early inflammatory reactions of importance in the normal course of healing. Studies on connective tissue in growth into subcutaneously implanted ivalon sponges also showed a relation between the intensity of the inflammatory reaction and the rate of regeneration Thus a sponge inserted atraumatically after having been soaked in physiological saline was seen to be infiltrated to a smaller extent by connective tissue than one inserted under considerable tissue injury and soaked in distilled water (EDWARDS PERNOKAS & DUNPHY 1957)

Previous workers seem to have agreed that administration of glucocorticoids to animals cause a considerably inhibited production of collagen This has been shown by both histological and biochemical

Table 3

Tensile strength of linear wounds not significantly decreased in the treated groups Number of animals in brackets

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treated with hydrocortisone, sodium salicylate or phenylbutazone in doses that merely altered the water content of the granulation tissues This suggests that the dosage in question does not cause the collagen fibrils to change much or be greatly reduced in number.

Summary

The effects of administration of hydrocortisone, sodium salicylate, phenylbutazone or prednisone on the production of granulation tissue have been investigated These drugs act by reducing the total amount of tissue produced, though the amount of dry, defatted tissue is less affected No significant reduction was observed in collagen content, expressed as hydroxyproline

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investigations RAGAN *et al* (1949), for instance, found production of all the connective tissue elements in wound tissue to be markedly inhibited during treatment with cortisone On the whole, the histological picture of healing under glucocorticoid treatment seems to be characterised by a greatly inhibited fibroblast reaction, a reduced number of collagen fibrils and a scant exudate, as well as few abnormal blood vessels (SPAIN *et al* 1953, ALRICH *et al* 1951, ZOGER 1952)

ROBERTSON & SANBORN (1958) noticed a reduced production of collagen in carrageenin provoked granulomas from cortisone treated animals TRNAVSKY, TRNAVSKA & MALINSKY (1962) arrived at the same result on examining the walls of turpentine provoked abscesses in prednisone treated rats

PERNOKAS, EDWARDS & DUNPHY (1957) found that, after implantation of ivalon sponges, collagen production was inhibited by cortisone doses that had no influence on the amount of dry tissue LAPIERE & CAUWENBERGE (1960) arrived at a similar result by treating rats with salicylate This contrasts with our observations reported here, as shown in table 2 The discrepancy is presumably to be explained by the different experimental techniques, as collagen production is known to proceed differently in a subcutaneously implanted ivalon sponge and in open granulation tissue The collagen percentage in such a sponge will always lie at a lower level than that in open granulation tissue of the same age and the collagen fibrils will never attain the same thickness as in an open wound (EDWARDS PERNOKAS & DUNPHY 1957) It is thus possible that somewhat defective collagen synthesis will occur even in a sponge from an untreated animal and that this is more susceptible to the influence of a harmful agent than the normal production Similarly, collagen synthesis does not proceed entirely along physiological lines in carrageenin provoked granulomas because carrageenin besides stimulating the production of collagen also promotes its breakdown (JACKSON 1957, WILLIAMS 1957)

The results of a quantitative investigation of the hydroxyproline in wound tissue indicate the total amount of mature collagen and its precursors Thus, this investigation does not serve to elucidate whether an inhibited production of mature collagen fibrils occurs while at the same time the total amount of collagen is normal It has been plainly demonstrated that the tensile strength of a wound is impaired in response to treatment with glucocorticoids (SAVLOV & DUNPHY 1954, HOWES *et al* 1950) As moreover, the strength of a wound is an indication of its content of mature collagen fibrils there is much evidence to suggest that collagen production proceeds abnormally if glucocorticoids are given in sufficient doses It should be noted in this connection that measurements of the tensile strength (table 3) showed no significant impairment in animals

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From the Department of Pharmacology, University of Copenhagen
(Professor Knud O Møller M D)

Effect of Ascorbic Acid on Formation of Granulation Tissue in Open Wounds

By

[illegible] Jørgensen

(Received June 29 1962)

The effect of ascorbic acid on collagen production and the healing of wounds has been the subject of numerous investigations, which have all shown that deficiency of this vitamin has a complex effect on regenerative processes

Many previous publications about the production of collagen and granulation tissue in vitamin C deficiency were based on studies of alterations in such tissue as carrageenin provoked granulomas, as well as in granulation tissue produced by subcutaneous implantation of ivalon sponges The work reported here aimed at throwing light on the effect of ascorbic acid deficiency on open granulation tissue produced by RUDAS's (1960) technique, which permits quantitative measurements of the granulation tissue produced in open wounds without admixture of other tissue this possibility of quantitative measurements has not been present in previous studies on open wounds

Methods

Female guinea pigs weighing about 300 [illegible] were used

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Hexosamine determinations were made by BLIX's (1948) modification of ELSON & MORGAN's method.

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Table 2

Hydroxyproline and collagen content of granulation tissue Hydroxyproline expressed as mg hydroxyproline/100 g dry, defatted tissue collagen as g collagen/100 g dry defatted tissue and total amount of collagen per granulation tissue in each animal number of animals

	n	Hydroxy proline	Collagen		Total collagen formed
		mg/100 g	g/100 g	s e m	mg
Control	16	2805	20 92	0 75	24 69
Scorbutic	15	520	3 88[1])	0 20	8 11
Partial[2]) scorbutic	15	1245	19 29[1])	1 03	14 [illegible]

[1]) Significantly different from control ($P < 0.001$)
[2]) Animals injected with ascorbic acid on the last two days before being killed

Discussion

Few workers have studied the amount of granulation tissue in scurvy An increased amount of dry granulation tissue has been found in scorbutic animals after subcutaneous implantation of ivalon sponges (CHEN & POSTLETHWAIT 1961) In contrast, HUGHES & KODICEK (1960) stated that the amount of granulation tissue in healing tendons was reduced in the scorbutic state DUNPHY, UDUPA & EDWARDS (1956) have reported an abnormally scant production of granulation tissue in open wounds of scorbutic animals, without, however, having performed strictly quantitative measurements of the amount of tissue produced

The healing processes in deep-seated tissues are only to a limited extent similar to those in open granulation tissue The reduced amount of tissue found in tendons and the increased amount in ivalon sponges are therefore not directly to be compared with our results presented here Our finding of a greatly increased production of both wet and dry, defatted granulation tissue in scurvy (table 1) is in fact, surprising, as agents that inhibit healing generally manifest themselves by reducing the amount of tissue (VAN CAUWENBERGE & LECOMTE 1957 a, b, RUDAS 1960, JORGENSEN 1962) The work under review affords no basis for pronouncing with certainty on the biological mechanism underlying the increase in amount of granulation tissue in ascorbic acid deficiency The intensely haemorrhagic appearance of the tissues as well as the raised contents of hexosamine and water, may well indicate increased exudation, which could contribute largely towards the occurrence of an increased amount of tissue A similar rise in water and hexosamine contents is also seen in ordinary skin tissue of scurvy (PERSSON 1953)

The raised hexosamine content may, however, be due to altered con-

Table 1

The weights of the wet and dry defatted tissue expressed in milligrammes hexosamine in milligrammes per 100 g dry, defatted tissue and the relative water contents

	n	Weight of wet tissue		Weight of dry defatted tissue		Relative water content		Hexosamine	
		mg	s e m	mg	s e m	g/100 g	s e m	mg/100 g	s e m
Control	16	683	31	118	5	478	11	872	20
Scorbutic	15	1340¹)	65	209¹)	10	535¹)	10	1127¹)	35
Partial*) scorbutic	15	982¹)	109	160¹)	13	491	16	992¹)	20

¹) Significantly different from control ($P < 0\ 001$)
*) Animals injected with 50 mg ascorbic acid on the last two days before being killed

Results

On removal of the granulation tissue 8 days after insertion of the rings, gross changes were plainly seen in the scorbutic tissues, which were abundant, haemorrhagic and friable

Table 1 records the weights of the wet and dry, defatted granulation tissue as well as its water and hexosamine contents The water content is expressed as grammes water per 100 g dry, defatted tissue Below this will be called the relative water content Hexosamine is expressed as milli grams hexosamine per 100 g dry, defatted tissue The table indicates a considerable difference between the scorbutic and the control group in the weights of wet and dry, defatted tissue as well as in the hexosamine and relative water contents (P for all these values $<0\ 001$) The short-term treatment with ascorbic acid changed all the values in the direction of the normal, most rapidly the relative water content which no longer differed significantly from the relative water content of normal granulation tissue The other values continued to be highly abnormal ($P < 0\ 001$)

Table 2 shows the hydroxyproline and collagen contents of granulation tissues An appreciable reduction is seen in the collagen contents of both scorbutic groups ($P < 0\ 001$) Owing to the pronounced increase in the amount of tissue in the scorbutic animals, the collagen percentage is no reliable indication of the quantity of collagen produced Therefore, the table records both the percentage of collagen and the total amount of this in the granulation tissue of each animal We see in the table that the total amount of collagen produced was also considerably reduced in the two scorbutic groups, even though the difference was somewhat less than for the percentage collagen contents

that collagen production proceeds normally, but is masked by a "dilution" of the hydroxyproline, due to the increased amount of tissue Comparison of the percentage values for collagen with the values for the total amount of collagen produced in the granulation tissues (table 2) shows that this in fact does play a part It is seen, however, that the changes in the amount of tissue cannot account for the entire reduction in collagen content of scorbutic tissue

An inhibited production of hydroxyproline in granulation tissue is claimed to be a sensitive sign of vitamin C deficiency (GOULD & WOESSNER 1957) However, our investigation has shown that the amount of tissue as well as the contents of hexosamine, and water change to the same extent as the content of hydroxyproline and, like this, alter rapidly towards normal in response to short term administration of ascorbic acid The relative water content responds the fastest, this having already become normal after the short term ascorbic acid treatment

Summary

The effect of ascorbic acid deficiency on the production of granulation tissue has been studied Ascorbic acid deficiency gives rise to a considerable increase in the amount of tissue produced, the relative water content and the hexosamine content of the tissues whereas the hydroxyproline content is greatly reduced Administration of vitamin C for 2 days causes a rapid change of the values towards normal The values for water change the most rapidly

Acknowledgements

This investigation was carried out in The Laboratory for Investigation of the Pharmacology of the Connective Tissue with a grant from the Danish National Association against Rheumatic Diseases

The author thanks Miss Lene Clausen for excellent technical assistance

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centrations of acid mucopolysaccharides Thus, it has been shown that scorbutic granulation tissue contains increased amounts of hyaluronic acid (ROBERTSON & HINDS 1956), there is also much evidence to suggest that the synthesis of chondroitin sulphate is inhibited (KODICEK & LOEWI 1956, HUGHES & KODICEK 1960)

An appreciable inhibition of collagen production in scorbutic tissue has been described by various workers DUNPHY, UDUPA & EDWARDS 1956, GOULD & WOESSNER 1957, GOULD 1958, CHEN & POSTLETHWAIT 1961) It is stated, however, that it is only the rapid collagen production in granulation tissue that depends on the presence of ascorbic acid The slow collagen production, on the other hand, which takes place continuously as part of the dynamic equilibrium of the organism, is believed to be independent of the presence of ascorbic acid (GOULD 1961) As all workers have found a small, though measurable, content of collagen in scorbutic granulation tissue, it has been thought possible that this might be due to contamination by collagen from the surrounding tissue In the light of the theory about collagen production independent of ascorbic acid, GOULD is of the opinion that the low hydroxyproline concentration observed in scorbutic granulation tissue is due, not to contamination but to the fact that a slow, ' basal" production of collagen, independent of the presence of ascorbic acid, takes place in granulation tissue Using RUDAS' technique, we find small, but by no means negligible amounts of collagen in scorbutic granulation tissue (table 2) As by this technique it is possible to obtain tissue with no contamination from the surroundings our results are consistent with GOULD's hypothesis

Nothing certain is known about the mechanism of reduced collagen production in scorbutic granulation tissue However, the defect seems to be due, at least in part, to deficient hydroxylation of proline into hydroxyproline (GOULD & WOESSNER 1957, CHEN & POSTLETHWAIT 1961) In agreement with the theory of the independent collagen production the hydroxylation in the body seems, however, on the whole to proceed normally during the course of scurvy (MITOMA & SMITH 1960)

We cannot say for certain whether the pathological ground substance seen in scurvy plays any part in the altered collagen synthesis In this connection it is a matter of some interest that JØRGENSEN & SCHMIDT (1962) found normal wound strength, and thus presumably normal collagen production, in mice treated with oestrogenic hormones This treatment gives rise to a considerable accumulation of hexosamine and water in the skin (SCHMIDT 1958) Accordingly, considerable changes can take place in the ground substance without concurrent alterations in collagen production

As the amount of tissue increases appreciably in scurvy, it is possible

that collagen production proceeds normally, but is masked by a "dilution" of the hydroxyproline, due to the increased amount of tissue Comparison of the percentage values for collagen with the values for the total amount of collagen produced in the granulation tissues (table 2) shows that this in fact does play a part It is seen, however, that the changes in the amount of tissue cannot account for the entire reduction in collagen content of scorbutic tissue

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Summary

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From the Biological Department the Pharmaceutical Institute
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Purification of the Main Kinin of Human Urine

By

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(Received June 20 1962)

A method has previously been described (BRISEID JENSEN 1958 BRISEID JENSEN & SUND 1959) for the purification of substance Z (WERLE & ERDOS 1954 GOMES 1955 WALASZEK 1957) now usually called human urinary kinin (GADDUM & HORTON 1959 HORTON 1959) The method was based on adsorption of the substances in urine stimulating the rat uterus on the carboxylic acid resin Amberlite IRC 50 and elution of the active principles with hydrochloric acid The acid elution however often gave poor recovery and in the work reported here we have accordingly attempted to determine whether an elution at alkaline pH as originally proposed by GADDUM & HORTON (1959) would improve the efficiency of the method At the same time the subsequent stages of the purification method were altered to make the procedure simpler and quicker The individual steps of the modified method have been tested to elucidate their contribution to the overall loss of activity A considerable loss of activity in one of these steps proved to be due to the existence of a second fraction stimulating the rat uterus

The final active concentrate was considered pure enough for a pharmacological and paper chromatographic comparison with bradykinin (BRISEID JENSEN & VENNEROD 1962)

Technique

A *Materials*

I *Standard preparation and urine* As standard preparation we used the same crude extract as previously (BRISEID JENSEN & SUND 1959) Substandards were prepared by the same method and compared with the primary standard in (2 + 2) assays on the isolated rat uterus

Pooled 24-hour urines were used in small scale experiments usually from 4 individuals in large scale experiments from 4-40 individuals If

the urine had to be kept for more than a 24-hour collecting period before use, it was frozen and stored at −17° Normally active solutions were assayed by "bracketing" the unknown between two standard doses, the standard dose ratio usually being 3 2 For special solutions (2 + 2) assays were used

II *Ion-exchange resin* The carboxylic acid resin Amberlite CG 50 (100–200 mesh), pre-treated as previously described, (BRISEID JENSEN & SUND 1959) was used The weights given for the resin in this paper refer to the free acid form dried to constant weight at 105°

III *Concentration apparatus* The active solutions were concentrated at 30° under a pressure of 5–8 mm Hg in a fixed condenser rotary film vacuum evaporator, A Gallenkamp & Co Ltd , England

IV *Chemicals* The reagents used were throughout of analytical or chromatographic grade

B *Purification Procedure*

The first stages of the purification method were as previously described (BRISEID JENSEN & SUND 1959) From the point at which the Amberlite CG-50 with the active principles adsorbed was washed free from acetic acid with distilled water, the procedure is new However, in order that the process may be more easily followed, the initial steps are also described below The figures there refer to a 50-litre batch of urine

I *Adsorption of activity* The urine (its pH being first adjusted to 6 0 ± 0 1) was mixed with 50 litres of water To this were added 200 g of Amberlite CG-50 pre treated and buffered to pH 6 0 ± 0 1, as previously described (BRISEID JENSEN 1958) The mixture was stirred vigorously for about 30 minutes

II *Washing* The resin was allowed to settle, most of the supernatant fluid could be decanted and discarded The rest of the fluid was sucked through a 15 cm diameter sintered immersion filter of porosity 3 (Baird & Tatlock) connected with a water pump The resin was then stirred in 5 litres of distilled water for a few minutes and the water was sucked off This procedure was repeated three times Next came washing with 1 N acetic acid, 4 × 3 litre portions, the mixture being stirred each time for about 15 minutes Then the resin was washed with distilled water to remove acetic acid, 7 × 3 litre portions of water being used

Finally the resin was stirred for about 30 minutes with 5 2 litres of 0 3 N sodium hydroxide (7 8 mEq per g resin), resulting in a pH of 9 2–9 7 in the supernatant The practically inactive, yellowish fluid was sucked off

and discarded Then the residue was washed with about 5 litres of distilled water, which was also sucked off and discarded

III *Elution of active material* The pH of the supernatant fluid was then brought to about 12 by stirring for half an hour with 2 litres of 0 3 N sodium hydroxide (3 mEq per g resin) The fluid, which contained nearly all the activity, was sucked off, and concentrated hydrochloric acid was added to give a pH of about 3

IV *Concentration and salt removal* The active solution was concentrated at 30° under reduced pressure (5–8 mm Hg) on the rotating evaporator to about 500 ml, and concentrated hydrochloric acid was added to a pH of about 1 5 Then the solution was saturated with sodium chloride and shaken twice with 500 ml of freshly distilled n-butanol The water phase was discarded, and the combined butanol fractions were extracted with 3 × 300 ml of distilled water The combined water fractions were concentrated to dryness at 30° under reduced pressure (5–8 mm Hg) on the rotating evaporator The active substance was eluted with 20 ml and then with 4 × 10 ml of concentrated acetic acid The combined acetic acid fractions were filtered through filter paper, and the acid was then quickly removed on the rotating evaporator, as described above The last traces of water and acetic acid were then removed at room temperature at a low pressure (0 005–0 01 mm Hg)

The concentrate was kept over silica gel in evaporated desiccators at room temperature

Comments on the Technique

A *Purification Procedure*

I *Washing* The acetic acid washing of the Amberlite CG-50 with the active material adsorbed has been discussed previously (BRISEID JENSEN & SUND 1959) If pH was then raised to 9 2–9 7 by addition of sodium hydroxide to the resin, the active substance was not eluted, this procedure was adopted to increase the efficiency of the washing now being carried out at both an acid and an alkaline pH Only traces of active material were lost through the sodium hydroxide washing (0 5–2%), even when the pH was raised to about 10, the losses were small

II *Elution of activity* GADDUM & HORTON (1959) showed that the activity could be eluted from Amberlite CG 50 at pH 9 5 by using a 0 2 M phosphate buffer In order to use a buffer system that could be transferred to sodium chloride by addition of hydrochloric acid (the sodium chloride so formed being useful in the subsequent procedure) we eluted instead with a 0 3 M sodium carbonate bicarbonate buffer The efficiency

of the elution was satisfactory, but the presence of free carbonic acid in the solution rendered its vacuum concentration difficult As is evident from the procedure description above, the buffer elution was accordingly omitted, and an elution with 0 3 M sodium hydroxide was used If the pH was brought to about 12, almost all of the adsorbed active principle was eluted from the resin If another elution was carried out, this time with the same volume of 0 01 M sodium hydroxide, the recovery was only increased by a few per cent The eluate acidified to pH 3 was fairly stable, after 6 days at +4° no loss of activity could be demonstrated

III *Concentration* In the purification method described previously (BRISEID JENSEN & SUND 1959), the active solutions were concentrated at a pressure of 18–22 mm Hg in a "Quickfit" Circulatory Cyclone Evaporator operated by steam Only small losses of activity during concentration could then be demonstrated The concentration procedure, however, was complicated by occasional splashing of the active solution, with loss of activity into the receiving flask We therefore replaced the Cyclone Evaporator by a rotating evaporator operated under a pressure of 5–8 mm Hg at 30° The loss of activity by concentrating in that way was negligible Although the distillation capacity of the rotating evaporator was far less than that of the cyclone evaporator (about 500 ml against 2000 ml per hour), the modification of the method also markedly reduced the volume of active solution to be concentrated

IV *Butanol and water extractions* GOMES (1955) extracted the material in urine stimulating rat uterus with n-butanol, in order to prepare a crude standard substance To transfer quantitatively the activity to the alcohol by shaking it proved necessary, as proposed by CLARK, WINCKLER, GOLLAN & FOX (1954) for the extraction of angiotonin, to acidify the urine to pH 1 5 and then to saturate it with sodium chloride GADDUM & HORTON (1959) also found that the butanol extraction procedure gave a total recovery of activity from urine In our method however, when the active concentrated solutions were shaken with freshly distilled n butanol, the losses were some 20 60% No further loss of activity occurred when the active substance was transferred from the butanol phase back to water

The effectiveness of the butanol extraction could not be improved by increasing the number of extractions Most of the recoverably activity passed over into the first butanol fraction, and only small additional quantities of active material could be transferred by shaking three or four times with the alcohol When the acid sodium chloride saturated water phase was examined, most of the residual activity could be demonstrated there The total loss of activity during the butanol extraction procedure was accordingly small (see Discussion)

V *Salt removal* Most of the salts present were left behind during the butanol extraction When the active solutions saturated with sodium chloride were shaken at pH 1 5 with n butanol, about 0 3% of sodium chloride was found in the alcohol (3 g in the 1000 ml butanol used for a 50-litre batch of urine) When we attempted to improve the result by drying the butanol phase over sodium sulphate before the sodium chloride determination, the quantity of salt found only decreased to about 0 1% An acetic acid extraction of the active principle from the residue obtained by concentrating the water shakings from butanol to dryness at 30° proved more effective About 0 05% of sodium chloride was found in the acetic acid (30 mg in the 60 ml acid used for a 50-litre batch of urine) A total loss of activity of about 10% was found for the combined processes of water concentration and acetic acid extraction

Results and Discussion

One of the reasons for altering the original purification procedure for human urinary kinin (BRISEID JENSEN & SUND 1959) was to make the method simpler and quicker This object can be said to have been achieved, the whole procedure for a 50-litre batch of urine being now carried through in 2 3 days as against 4–5 days previously The other reason for making alterations in the method was to discover whether the effectiveness of displacing the active principles from the ion exchanger (Amberlite CG-50) could be improved on replacing the acid by an alkaline elution It has previously been suggested (BRISEID JENSEN & SUND 1959) that the occasionally low recovery of activity from the ion exchanger, observed when hydrochloric acid was used for elution might be due to more than one active kinin being present The sharp peak of the acid elution curve, and the fact that the recovery of activity could be greatly increased by subsequent elution at pH about 9 gave evidence for such a view Table 1 shows an example of a purification experiment carried through with a 50-litre batch of urine Recoveries are given for the different stages of the process calculated on the basis both of the original activity and the activity of the preceding stage The activity of the urine in this particular experiment dropped too quickly to render an assay possible It has previously been shown (BRISEID JENSEN & SUND 1959) that the activity of urine falls somewhat quickly at pH 6 if the urine has not been stabilized by heat treatment at slightly acid pH

The figure for the activity of the sodium hydroxide eluate refers to an assay of the eluate acidified to pH 3 and kept over night at 4°

The butanol extraction described here was carried through in a somewhat different manner from the proposed procedure Instead of shaking

Table 1.

Recoveries of the rat uterus stimulating principles of urine at different stages of the purification procedure

The figures refer to an experiment with a 50 litre batch of urine, for further details see text

Stages of purification	Units	Activity % recovery referring to eluate from Amberlite CG 50	preceding stage
1 Sodium hydroxide eluate from Amberlite CG 50	147000	100	100
2 Eluate concentrated	143000	97	97
3 Combined water extracts from butanol extract of the concentrated eluate	72000	49	50
[3x Water phase after butanol extraction]	[43000]	[29]	[30]
4 Combined water extracts concentrated to dryness, acetic acid extract of the residue concentrated to dryness	68000	46	94

the water phase twice with 500 ml of butanol, we used 300 ml, 200 ml and 200 ml of butanol for the extraction

Table 1 shows that the recoveries were good at all stages of the new purification procedure, except for the butanol extraction Other experiments gave the same result However, the activity was not lost, most of it could be found in the residual water phase The question was now whether that activity represented another rat uterus stimulating principle or whether inactive substances from the urine could inhibit the transfer of active substance from water to butanol To study this question, model experiments were carried out with

1 A previously prepared sample of human urinary kinin
2 *Synthetic bradykinin*[1])
3 The residual water phase (containing rat uterus stimulating activity) saturated with sodium chloride after butanol extraction of active substance brought to pH 1 5 by addition of hydrochloric acid

The human urinary kinin test substance was prepared by the procedure described in this paper

The reason for examining bradykinin was that comparative paper chromatographic and pharmacological experiments with bradykinin and a human urinary kinin prepared as described here gave evidence that the two substances must be identical or closely related (BRISEID JENSEN &

1) Bradykinin *ampoules containing 10 or 100 µg/ml* were kindly furnished by Sandoz A G, Basel, Switzerland

Table 2.

Recoveries by butanol extraction from water of bradykinin and of rat uterus stimulating principles isolated from human urine

Test solutions
A 100 ml water rest phase after butanol extraction brought to pH 1 5 (Corresponding [illegible] stage 3x in table 1)
[illegible]
6000 units of urinary kinin
E. 100 ml sodium chloride saturated hydrochloric acid of pH 1 5 + 100 µg bradykinin equivalent [illegible] 7500 units of urinary kinin
Activities are given as units of human urinary kinin, for further details see text.

Test solution	Activities				
	Start	Recovered		Left in water phase	
	units	units	%	units	%
A	3000	950	32	1100	37
B	6000	2500	42	1900	32
C	6700	4300	64	1000	15
[illegible]	6000	3500	58	950	16
E	7500	5300	71	<50	<1

VENNEROD 1962) Table 2 shows the design and the results of the model experiments

The test solutions (100 ml) were each shaken twice with 100 ml butanol The transferred active materials were recovered from the combined butanol fractions by shaking three times with 70 ml water Aliquots for assay were concentrated to dryness at 30° and about 15 mm Hg on a rotating evaporator and then dried at 0 01–0 05 mm Hg at room temperature

Table 2 seems to indicate that two (or more) rat uterus stimulating principles were present in the extracts The table clearly shows that the residual water phase activity could only be partly extracted with butanol, whereas most of the previously prepared urinary kinin and all the bradykinin were transferred from acidified water solutions This means that a concentrate of urinary material prepared by the proposed method will contain some of the active material not easily transferred to the butanol The concentrate used in these experiments contained about 30% of the unknown material in terms of rat uterus stimulating activity, but that figure may well vary according to the relative amounts of the two active fractions in the urine used

The active principle in the residual water phase could be a substance preexisting in the urine, but it could also be a product of the urinary

kinin resulting from the purification procedure Some previous papers seem to indicate that the first alternative may be the right one Thus previous chromatographic work (WALASZEK 1957, GOMES 1957, BRISEID JENSEN 1958) gave some evidence that at least two active fractions were present in urine extracts Further, the strongly varying recovery of activity that could be demonstrated by hydrochloric acid elution of Amberlite CG-50 (BRISEID JENSEN 1958, BRISEID JENSEN & SUND 1959) could be due to the presence of more than one rat uterus stimulating sub stance in urine, one being less extractable by acid

On the other hand both GOMES (1955) and GADDUM & HORTON (1959) reported on good recoveries of activity by butanol extraction, the latter authors using as material both urine and eluates from Amberlite CG 50 obtained by alkaline elution (phosphate buffer) at pH 9 Some experiments carried out by us using elution of the activity from Amberlite CG 50 at pH 9 5 (carbonate buffer) also gave eluates from which most of the activity could be shaken into butanol The last-mentioned results seem to suggest that the activity, which was only slowly transferable to butanol by the proposed procedure, might be a transformation product of the urinary kinin However, the presence of rather small quantities of the less butanol soluble principle might also explain the high recoveries in those experiments In order to discover whether an elution at pH about 12 could alter bradykinin, an attempt was made to isolate a known quantity of bradykinin, dissolved in water, according to the method stated Table 3 shows the result of the experiment

The loss of activity from initial solution to the sodium hydroxide eluate

Table 3

Recoveries of bradykinin at different stages of the purification procedure

The figures refer to an experiment with 200 µg bradykinin equivalent to 14900 units of human urinary kinin dissolved in 9 litres of water
Activities are given as units of human urinary kinin

Stages of purification	Units bradykinin	Activity % recovery referring to	
		water solution of bradykinin	preceding stage
1 Water solution of bradykinin	14900	100	100
2 *Sodium hydroxide eluate from Amberlite CG 50*	11100	75	75
3 Eluate concentrated	11100	75	100
4 Combined water extracts from butanol extract of the concentrated eluate	8100	54	73
[4x Water phase after butanol extraction]	[<500]	[<3]	[<6]

was due to a somewhat rapid decline in activity of the aqueous bradykinin solution, a fall in activity was noticed during assay of the solution Only small amounts of active substance could be detected in the residual water phase after butanol extraction, showing that bradykinin was not transformed into a less butanol soluble compound during the procedure

If it is assumed that bradykinin is identical with the substance easily extracted by butanol the results give support to the view that the residual water phase activity of table 1 was due to a substance preformed in the urine However, as the experiments were carried out with an aqueous solution of bradykinin and without urine, the possibility cannot be excluded that a transformation can occur in presence of substances from urine that are not extracted from the ion exchanger at a pH of 9 5, but are at a pH of 12 For a final conclusion to be possible, a purification of the active principle of the residual water phase and its comparison with urinary kinin and bradykinin should be carried out

Summary

A method has been described for preparing a concentrate of the main kinin of human urine, it is a modification of a purification procedure previously reported (BRISEID JENSEN & SUND 1959) and includes batchwise adsorption of the active principles on Amberlite CG 50 (100–200 mesh), washing the resin with water, 1 M acetic acid and sodium hydroxide at pH about 9 5, elution of the activity with sodium hydroxide at pH about 12, acidification with hydrochloric acid to pH about 3 and concentration to a small volume under reduced pressure at 30°, removal of inorganic salts by transferring the activity to butanol at low pH, transfer of the active principles to water and concentration to dryness under reduced pressure at 30°, elution of the activity with acetic acid and concentration to dryness under reduced pressure at 30°

The main purpose of altering the purification method previously described was to improve the recovery of activity, first of all by a more effective elution of the Amberlite CG 50 The alterations made considerably increased the yield from the ion exchanger, but did not significantly increase the final recovery of urinary kinin However, the loss of activity was only apparant two (or more) different kinins being present in the eluate from the resin The first kinin could like bradykinin, be quantitatively transferred from water to butanol at low pH, whereas the second kinin fraction to a large extent remained in the water phase The final concentrate of the kinin easily extractable by butanol was considered pure enough for a pharmacological and chromatographic comparison with bradykinin (BRISEID JENSEN & VENNERÖD 1962)

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From the Biological Department, the Pharmaceutical Institute,
University of Oslo

A Pharmacological and Chromatographic Comparison of the Main Kinin of Human Urine with Bradykinin

By

Kjell Briseid Jensen and Anne Marie Vennerod

(Received June 20 1962)

Several previous papers have indicated that human urinary kinin is identical with or very closely related to bradykinin The urinary substance could be distinguished from the plasma kinin neither in pharmacological parallel assays (GOMES 1955, WALASZEK 1957, GADDUM & HORTON 1959) nor by chromatography (WALASZEK 1957) There is, however, some pharmacological and chemical evidence that the active principles are different (GOMES 1957) As the dissimilarities observed may be due to inhomogeneity of or impurities in one or both of the preparations used, we decided to compare synthetic bradykinin with a purified preparation of the main urinary kinin (BRISEID JENSEN & VENNEROD 1962) in both pharmacological and chromatographic tests

1. Technique.

A *Preparations of Human Urinary Kinin and Bradykinin*

The standard preparation of urinary kinin was the same crude extract as that used previously (BRISEID JENSEN & SUND 1959), sub-standards being prepared by the same method In the experiments reported here we used a concentrate of the main urinary kinin prepared as described in an earlier paper (BRISEID JENSEN & VENNEROD 1962) The activity of the preparation was determined on the isolated rat uterus

As bradykinin standard preparation we used synthetic bradykinin, which was available in ampoules containing 10 or 100 µg/ml[1]) The same bradykinin was also used as test substance in the experiments The rat uterus stimulating activity of 1 mg (1 unit) of the urinary kinin standard corresponded to 13 4 ng of bradykinin (2 + 2 assay)

In the paper chromatographic experiments bradykinin was not used as standard substance For practical reasons not only were the fractions containing urinary kinin

[1]) The bradykinin ampoules were kindly furnished by Sandoz A. G., Basel, Switzerland

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In the paper chromatographic experiments bradykinin was not used as standard substance For practical reasons not only were the fractions containing urinary kinin

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assayed against the urinary substance standard, but also the bradykinin + urinary kinin fractions as well as the pure bradykinin fractions.

In the pharmacological experiments no standard preparation was used, the *comparison* between the effects on different biological preparations being emphasized. Samples from the same batch of urinary kinin concentrate were accordingly used throughout and assayed against the same batch of bradykinin ampoules. The figures given for relative activities of bradykinin and urinary kinin in the pharmacological experiments were calculated on the basis of rat uterus assay, the relative strength of bradykinin and urine substance standard being known in advance (13.4 ng–1 unit).

B. *Pharmacological Assays.*

I. *Rat uterus.* Uteri from virgin rats weighing 140–190 g were suspended in de Jalon solution (GADDUM, PEART & VOGT 1949) maintained at 29–31° (±0.1°). The rats were injected with stilboestrol (10 µg/100 g) the day before they were killed. The uterus was generally used within a few hours of the animal's death or stored at +4° for one or two days before use. The muscle was attached to an isotonic frontal writing lever (SCHILD 1947) yielding a magnification of about 5. The load usually ranged from 0.6–0.9 g. The period of contact of active solution varied from 50 to 75 seconds. The interval between doses was fixed at 4 minutes. The bath was supplied with air. The experiments were done in an automatic assay apparatus from Casella Electronics Ltd., London. The bath volume was about 3 ml, and the fluid was changed by emptying the vessel and refilling from stock bottles. The washing between doses consisted in a fourfold change of bath fluid.

II. *Guinea pig ileum.* A guinea pig (usually weighing 200–300 g) was killed by a blow on the head and bled. A 3–4 cm segment of terminal ileum was suspended in Tyrode solution maintained at 36–37° (±0.1°). If spontaneous contractions were troublesome, the concentration of Ca^{++} in the solution was decreased by half. Atropine sulphate (0.1 mg/l) was added. The writing lever was as described above for the uterus, the load ranged from 0.7–0.8 g. The period of contact of active solution varied from 45 to 60 seconds, and the interval between doses was fixed at 4 minutes. A 10 ml organ bath was employed, in which the fluid could be replaced by means of upward displacement and overflow. Contractions were produced by manual addition of volumes ranging from 0.2–0.5 ml from 1 ml graduated syringes. A dose ratio of 1.5 was used and the concentrations of urinary kinin and bradykinin were about 2 units/ml and 20 ng/ml, respectively. Washing between doses was performed in two stages: washing for 10 seconds, an interval of 5 seconds and then washing for 5 seconds. The total volume of wash fluid was 50–60 ml from supply bottles of suitable height. The bath was supplied with air.

III. *Rat duodenum.* GADDUM & HORTON (1959) and HORTON (1959) showed that rat duodenum relaxes on addition of small quantities of kinins, they used comparative assays on that preparation and on rat uterus for the identification of kinins. The proximal 3–4 cm segment of duodenum of a rat weighing 150–250 g was suspended in de Jalon solution at 30° (±0.1°). A dose ratio of 2 was used and the concentrations of the urinary substance and bradykinin were about 0.2 units/ml and 4 ng/ml, respectively. For the rest, the apparatus and working conditions were as for the guinea pig ileum.

IV. *Rat blood pressure.* Male rats weighing 250–350 g were anaesthetized with a solution containing 4% w/v of sodium barbitone and 6% w/v of urethane (GADDUM & HORTON 1959): of this, 1 ml/100 g was injected intraperitoneally. The trachea was

cannulated, and artificial respiration was applied Blood pressure was recorded from a carotid artery (heparin in the polythene cannula) with a mercury manometer, and injections of the two test substances were made into the two femoral veins The bradykinin was diluted in 0 9% sodium chloride to about 5 μg/ml and the urinary substance to a concentration of 150–200 units/ml The volumes injected were 0 1 ml for the lower and 0 2 ml for the higher doses When 3 of the 6 series of a complete (2 + 2) assay were completed, the vein used for the bradykinin injections was used for the urinary substance and vice versa

C Chromatography

Descending chromatography with Whatman No 1 filter paper was used The chromatograms were run at room temperature (19–23°) for 12 to 23 hours The [illegible]

[illegible] (10 + 50 vol)

[illegible] + 50 vol)

3 Isopropanol, ammonia 25% w/v water (80 + 2 + 18 vol)

After chromatography the paper was dried in a stream of slightly heated air, and for every point of application of active substance cut into 5 cm broad parallel strips, which were again cut into 1 cm pieces The active substances were localized by assay on the rat uterus The 5 × 1 cm paper strips were eluted just before assay by shaking cautiously with 5 ml of de Jalon's solution and decanting the fluid

D Chemicals

The chemicals used were of analytical or chromatographic grade throughout

2. Results.

A. Pharmacological Assays

Some results of comparative assays of urinary kinin and bradykinin on different biological preparations are given in table 1 The figures recorded were obtained in (2 + 2) assays with 6 series of four randomized doses The statistical procedure described by SCHILD (1942) was used for the treatment of the results

Table 1

ng bradykinin equiactive with 1 mg (1 unit) of human urinary kinin in parallel assays Index of discrimination based on the rat uterus value

Biological effect	ng bradykinin (Fiducial limits ‰ P = 0 05)	Index of discrimination
Rat uterus stimulation	13 4 (94–106)[1]	–
Guinea pig ileum stimulation	9 7 (97–104)	0 7
Rat duodenum relaxation	14 8 (89–113)	1 1
Rat blood depressor effect	26 3 (83–120)	2.0

[1]) Results based on two combined (2 + 2) assays

Table 2.

Paper chromatography of bradykinin and human urinary kinin

Liquid system 1 N-butanol, acetic acid water (40 + 10 + 50 vol)

Bradykinin applied A quantity corresponding to 38 units of urinary kinin

Urinary kinin applied 50 units

Bradykinin + urinary kinin applied 38 + 50 units in terms of urinary kinin

Chromatogram run for 13½ hours

Fraction number	Recovery %		
	Bradykinin	Urinary kinin	Bradykinin + urinary kinin
1–3	–	–	–
4	–	–	–
5	–	6 0	5 5
6	–	2 0	–
7	2 5	18 0	7 0
8	31 5	18 0	22 5
9	9 0	12 0	16 0
10	–	1 0	–
11	–	–	–
12	–	–	–
13	–	–	–
14	5 0	–	–
15	–	–	–
16	–	–	–
17	–	–	–
18	–	not tested	–
19	–	not tested	–
20	–	not tested	–
21	–	not tested	–
22	–	not tested	–
23	–	not tested	–
24	–	not tested	–
25–27	not tested	not tested	–
Sum	48 0	57 0	51 0

B *Chromatography*

Tables 2, 3 and 4 give the results of typical examples of paper chromatographic experiments run in the 3 liquid systems used In all the experiments bradykinin and urinary kinin were run both alone in parallel and in mixture The results are calculated as percentage recoveries, values for recoveries less than 1 per cent being omitted Figure 1 gives the results of the chromatography in liquid system 1, in which the two active substances gave more distinct peaks than in the two other solvents used

Table 3

Paper chromatography of bradykinin and human urinary kinin

Liquid system 2 Hydrochloric acid 0.2 N, ethanol (50 + 50 vol)
Bradykinin applied A quantity corresponding to 56 units of urinary kinin
Urinary kinin applied 70 units
Bradykinin + urinary kinin applied 70 + 56 units in terms of urinary kinin
Chromatogram run for 14 hours

Fraction number	Recovery %		
	Bradykinin	Urinary kinin	Bradykinin + urinary kinin
1-16	not tested	not tested	not tested
17	–		–
18	–		–
19	–	–	–
20	–	–	–
21	–	–	–
22	4.5	1.0	15.0
23	22.5	21.5	44.5
24	11.0	21.5	14.0
25	1.0	3.5	4.0
26	2.5	2.5	1.0
27	1.0	1.5	–
28		–	–
29	–	–	–
30	–	–	–
31	–	–	–
Sum	42.5	51.5	78.5

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	Bradykinin	Urinary kinin	Bradykinin + urinary kinin
1–3	–	–	–
4	–	–	–
5	–	6.0	5.5
6	–	2.0	–
7	2.5	18.0	7.0
8	31.5	18.0	22.5
9	9.0	12.0	16.0
10	–	1.0	–
11	–	–	–
12	–	–	–
13	–	–	–
14	5.0	–	–
15	–	–	–
16	–	–	–
17	–	–	–
18	–	not tested	–
19	–	not tested	–
20	–	not tested	–
21	–	not tested	–
22	–	not tested	–
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24	–	not tested	–
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1-16	not tested	not tested	not tested
17	–	–	–
18	–	–	–
19	–	–	–
20	–	–	–
21	–	–	–
22	4.5	1.0	15.0
23	22.5	21.5	44.5
24	11.0	21.5	14.0
25	1.0	3.5	4.0
26	2.5	2.5	1.0
27	1.0	1.5	–
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9	9 0	12 0	16 0
10	–	1 0	–
11	–	–	–
12	–	–	–
13	–	–	–
14	5 0	–	–
15	–	–	–
16	–	–	–
17	–	–	–
18	–	not tested	–
19	–	not tested	–
20	–	not tested	–
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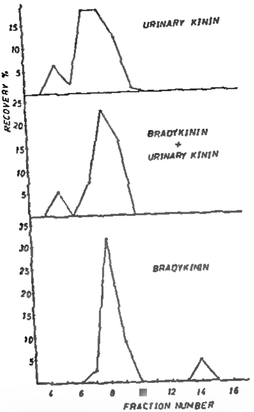

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3. Comments on the Results.

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Comparative assays on different biological preparations gave no evidence of qualitative differences between the concentrate of the human urinary kinin and the sample of synthetic bradykinin used The results thus agreed with previous ones from parallel assays with other preparations of bradykinin and the urinary substance (GOMES 1955, WALASZEK 1957, GADDUM & HORTON 1959)

The results shown in table 1 refer to representative examples from several experiments The index of discrimination given for the rat blood depressor effect which deviated significantly from 1, was found to be of the same order in other experiments However, the concentrations of the polypeptides were much higher than those used for the other pharmacologi-

Table 4.

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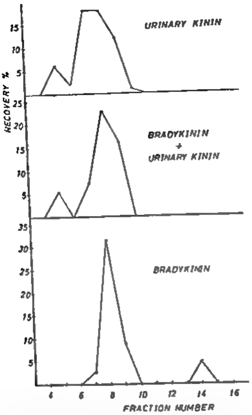
Liquid system 3 Isopropanol, ammonia 25% w/v, water (80 + 2 + 18 vol)
Urinary kinin applied 23 units
Bradykinin + urinary kinin applied 25 units + 23 units in terms of urinary kinin
Chromatogram run for 23 hours

Fraction number	Recovery %	
	Urinary kinin	Bradykinin + urinary kinin
1	2 0	2 0
2	4 0	2 5
3	8 5	2 0
4	3 0	2 0
5	4 0	1 5
6	2 0	1 0
7	3 0	1 0
8	4 0	3 0
9	3 0	7 0
10	1 0	4 0
11	–	–
12	–	–
13	–	–
14	–	–
15	–	–
16	–	–
17–34	not tested	not tested
Sum	34 5	26 0



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2	4.0	2.5
3	8.5	2.0
4	3.0	2.0
5	4.0	1.5
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7	3.0	1.0
8	4.0	3.0
9	3.0	7.0
10	1.0	4.0
11	–	–
12	–	–
13	–	–
14	–	–
15	–	–
16	–	–
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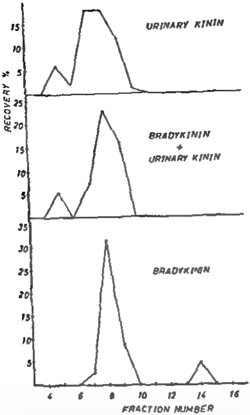

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A *Pharmacological Assays*

Comparative assays on different biological preparations gave no evidence of qualitative differences between the concentrate of the human urinary kinin and the sample of synthetic bradykinin used The results thus agreed with previous ones from parallel assays with other preparations of bradykinin and the urinary substance (GOMES 1955, WALASZEK 1957, GADDUM & HORTON 1959)

The results shown in table 1 refer to representative examples from several experiments The index of discrimination given for the rat blood depressor effect, which deviated significantly from 1, was found to be of the same order in other experiments However, the concentrations of the polypeptides were much higher than those used for the other pharmacologi-

cal preparations, and the presence of small quantities of other substances might well account for the deviation found in the index The bradykinin being only available in solution in ampoules, it seemed probable that stabilizing substances were present and could influence the assays In the rat blood depressor experiments we accordingly dissolved the urinary kinin in the contents of bradykinin placebo ampoules before diluting with 0 9% sodium chloride solution If parallel assays were run with the urine substance dissolved in 0 9% sodium chloride solution only, indices of discrimination of 4–5 could be established The bradykinin used being synthetic, and probably therefore relatively pure, it seems justifiable to suppose that inhomogeneity of or impurities in the concentrate of urinary substance might well cause the relatively smaller quantity of it necessary compared with that of bradykinin We do in fact know that another rat uterus stimulating principle could to some extent be present, being only incompletely removed during the purification procedure (BRISEID JENSEN & VENNERÖD 1962)

II *Chromatography*

I Liquid system 1 Table 1 and fig 1 show that both the bradykinin preparation and the urinary kinin used could be separated into two active fractions However, though the main zones of activity might well be identical from their positions, the small active zone of the urinary kinin could not be demonstrated in the bradykinin, and the faster running small active zone of the bradykinin could not be demonstrated in the urinary kinin In a mixed run of the two substances, the small bradykinin peak was found during assay of the fractions, but the peak recovery was less than 1% and the results have accordingly been omitted from table 1 and fig 1 (see Results, Chromatography) In other experiments, however, the bradykinin peak mentioned was also unmistakenly demonstrable in the mixed runs The presence of the urinary kinin, or rather of the impurities in it, might be the reason for the small zones of activity being rather diffuse in the mixed runs

The presence of the small active zone of urinary kinin was also confirmed in other experiments

II *Liquid system 2* The bulk of activity of the two kinins passed together and gave relatively distinct peaks, as is evident from table 2 They travelled considerably faster than in the butanol acetic acid system, the R_F-values being of the order of 0 8 against 0 3 As with the liquid system 1, a second peak could sometimes be demonstrated in the bradykinin runs It is evident from the figures given in table 2 that it was small, it was not clearly demonstrable in the mixed run of the kinins It could

however, be demonstrated in other experiments, both in single bradykinin runs and in mixed runs of the two substances

III *Liquid system 3* The isopropanol ammonia system proved to be less suitable for chromatography of the kinins The activity spread over several fractions, accordingly giving peaks of low activity However, the zones of activity of the two substances passed together in the alkaline liquid system (table 3) as they did in the acid systems, thus giving no evidence of dissimilarity between the two kinins

IV *Recovery of activity* The recoveries of activity will depend on several factors, of which inactivation during chromatography and loss through adsorption onto the filter paper, both during the run and in connection with the elution are probably the most important

In all the chromatographic experiments the activities of the solutions of the kinins used for application to the paper were checked in advance against the same standard urinary substance, as was to be used for assay of the fractions In the experiment recorded in table 2, the activity of 9 ng of bradykinin was, for some reason found to correspond to about 1 unit of the standard urinary kinin, the previously established ratio having been 13 4 ng to 1 unit However, the activity of the sample of the urinary kinin concentrate to be applied was likewise found to be greater than that of previously assayed samples from the same batch The recoveries for both kinins accordingly became of the same and usual order, namely, 45–60% Similar experiences in other experiments eliminated the possibility of an error having been made in weighing or dilution

Fractions that according to the results in tables 2, 3, and 4, were not tested for activity in the experiments illustrated, had proved to be inactive in other runs

4 Discussion.

Neither the pharmacological parallel assays on different biological preparations nor the paper chromatographic experiments gave evidence [illegible]

[illegible] the pharmacological assays did not deviate more from 1 than might be expected and the main activity peaks of the two kinins did not separate in the chromatographic experiments The results were thus consistent with the work of GOMES (1955), WALASZEK (1957) and GADD[illegible]

st[illegible] demonstrable in the synthetic bradykinin

(another secondary peak was found in the urinary kinin) Stabilizing substances in the bradykinin ampoules were not responsible for that effect Neither did the contents of placebo ampoules stimulate the rat uterus when applied at increasing concentrations, nor did an addition of actual amounts of the placebo substances affect the contractions produced by the urinary kinin

We did not arrive at the conclusion of GOMES (1957), that pharmaco logical parallel assays to some extent, and physicochemical separations in particular, showed the two kinins to be dissimilar However, during the testing of the individual steps in the purification procedure for the main urinary kinin (BRISEID JENSEN & VENNEROD 1962) we could demon strate considerable amounts of another material stimulating the rat uterus This seemed to vary considerably in quantity from one batch of urine to another It was to a large extent removed from our concentrate of urinary substance during the purification procedure, but might, if present in larger quantities in another type of urinary extract, well explain ■ detected qualitative difference between bradykinin and urinary kinin

Summary.

A comparison has been made between synthetic bradykinin (Sandoz, A G , Basel, Switzerland) and a purified preparation of the main kinin of human urine (BRISEID JENSEN & VENNEROD 1962) Paper chromatographic experiments with different solvents gave no evidence of any qualitative difference between the principal constituents of the substances used, the main zones of activity passing together Pharmacological parallel assays on different biological preparations (rat uterus, rat duodenum, guinea pig ileum, rat blood pressure) also supported the view that the two kinins are identical or closely related to each other

However, the paper chromatographic experiments showed that the bradykinin preparation used contained ■ small quantity of another slow contracting substance, and the concentrate of human urinary kinin small amounts of yet another active substance The second active fraction from the urinary kinin concentrate might be identical with an active fraction varying in amounts with the urine used, that was largely removed during the purification procedure (BRISEID JENSEN & VENNEROD 1962)

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The Concentrations of Morphine in the Brains of Rats Receiving Morphine and Neostigmine

By

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(Received August 3, 196?)

Several cholinergic agents, for instance neostigmine and physostigmine, potentiate the effect of centrally acting analgesics such as morphine and pethidine (SLAUGHTER *et al* 1940 a, b, c, KOMLÓS *et al* 1950, PÓRSZASZ *et al* 1951, SAXENA 1958, SCHAUMANN 1959) An explanation of this could be a higher concentration of morphine in the brain after a given dose when the analgesic is administered along with cholinergic drugs, as suggested by KNOLL, KOMLÓS & TARDOS (1953) The intention of the experiments recorded here was to elucidate whether neostigmine alters the distribution of morphine in rats in this way when the substances are given together intraperitoneally

Experiments and Methods

Adult male albino rats (115–205 g) were used Morphine tolerance in the rats were produced as previously described (JÓHANNESSON 1962) The normal rats are referred to below as non tolerant rats

Injection solutions and doses In all experiments morphine was given as an aqueous solution containing 37 5 mg/ml of the chloride Injections were given intraperitoneally Non tolerant rats were given 500 mg/kg the tolerant rats 700 mg/kg The rats normally survived these doses given alone

Neostigmine bromide was given as an aqueous solution containing 0 2 mg ml Injections were given intraperitoneally The dosage was 0 2 mg/kg The rats survived this dosage given alone

Experiments Non tolerant rats were injected with morphine alone or with morphine plus neostigmine in the doses mentioned above When both drugs were given to the same animal morphine was injected first and then immediately neostigmine from another syringe, the animals died 12–24 minutes after the injections (mean 18 minutes) Then the brain (including the brain stem and the cerebellum) was removed for determinations of morphine (see later)

To be able to evaluate any possible effect of neostigmine on the concentration of morphine in the brain, morphine was given alone to another group of rats These animals were killed by a blow on the head at times similar to the times of spontaneous death of animals in the first group (given both morphine and neostigmine) Thus we attempted to obtain the same mean time from giving the injections until the animals died either by drug action or were sacrificed

The same procedure was applied to groups of morphine tolerant rats Only the dosage of morphine in this series was 700 mg/kg to secure lethal action jointly of morphine and neostigmine (0 2 mg/kg) Spontaneous death after intraperitoneal injection of morphine with neostigmine occurred after 14–26 minutes (mean 18 minutes) The animals of a control group given morphine alone were allowed to live at approximately the same time

Determination of morphine was performed by the method of PÆRREGAARD (1957), modified by MILTHERS (1959), giving an average recovery of about 95% All results are expressed as μg morphine chloride per gram brain

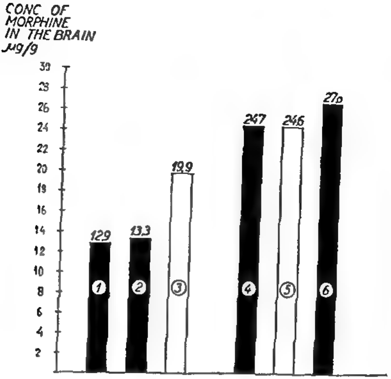
Descending paper chromatography was performed as described by BROSSI, HÄFLIGER & SCHNIDER (1955), with a mixture of amylene hydrate/*di n* butyl ether/water in the ratio 80/7/13 Whatman paper nr 1, buffered with phosphate buffer to pH 6 3, was used Paper chromatography was continued for 16 hours at 22°C, morphine was then eluted and determined as usually The R_F value of morphine was between 0 06 and 0 07

Two rats were killed without any previous administration of drugs Their total brains were examined for morphine and gave no reading Neostigmine bromide was added to a homogenate of these brains (200 μg/5 ml) The sample was taken through the analytical procedure and also gave no reading

Methodological Considerations

In unpublished investigations on concentrations of morphine in the brains of non tolerant rats, a higher concentration was found when morphine and neostigmine had been given intraperitoneally than when morphine was administered alone Later experiments revealed these results to be erroneous When paper chromatography was included in the analytical procedure, the difference disappeared (fig 1) However, in the morphine tolerant rats this difference never existed (fig 1) In a preliminary experiment one group of non-tolerant rats was injected intravenously with morphine and neostigmine, and another group of non-tolerant rats received the same dose of morphine alone by intravenous injection Morphine was found (paper chromatography not included) at the same concentration in the brains of rats in both groups Thus, the analytical error referred to above did probably not arise when the drugs were administered intravenously to non tolerant rats

In earlier experiments (MILTHERS 1961), and from some of the animals in our own experimental groups, morphine determinations on brains from rats given morphine alone, yielded the same results whether paper chromatography was included in the analytical procedure or not



... the brains of
analytical procedure *white columns* refer to experiments in which paper chromatography was not included These results were erroneous in non tolerant animals
Columns 1–3 indicate the mean concentration (µg/g) of morphine in the brains of *non tolerant* rats
... *of tolerant* rats
... gmine
... e

Eight rats given 0 2 mg/kg neostigmine by intraperitoneal injections were *killed 20 minutes later The brains were taken through the analytical* procedure (without paper chromatography) They did not yield any reading Four rats were given the same dosage and killed 45 minutes later From the brains of two of these animals we obtained readings indistinguishable from those due to morphine in the polarogrammes

Results

Twelve non-tolerant rats were injected with morphine (500 mg/kg) and neostigmine (0 2 mg/kg) simultaneously This dosage was lethal to all animals Death occurred 12–24 minutes after the injections (mean 18 minutes) The concentrations of morphine in the brains from these animals are shown in table 1, column 1 The mean concentration was

Table 1

Morphine concentrations (µg/g) in the brains of morphine tolerant and non tolerant rats injected intraperitoneally with morphine or morphine and 0 2 mg/kg neostigmine

Non tolerant rats		Morphine tolerant rats	
Morphine 500 mg/kg + neostigmine	Morphine 500 mg/kg	Morphine 700 mg kg + neostigmine	Morphine 700 mg/kg
20 9	11 3	23 5	33 7
15 0	12 4	36 3	22 9
12 1	13 7	24 9	33 0
7 0	14 7	24 6	19 3
13 6	8 7	16 1	26 7
10 5	13 7	26 6	21 8
13 5	13 7	37 1	29 4
13 4	12 7	–	16 2
14 4	14 9	–	17 7
13 4	13 6	–	22 0
12 8	10 3	–	32 6
13 0	14 7	–	20 7

12 9 µg/g For comparison twelve non tolerant rats were injected with 500 mg/kg morphine only As previously mentioned, this dosage is not lethal to rats in acute experiments The animals were killed at times similar to the times of death in the first group The brains were analysed for morphine (table 1, column 2) the concentrations found did not differ from those in the brain of rats given morphine and neostigmine, the mean was 13 3 µg/g

A similar scheme was used for the morphine tolerant rats One group of seven animals received 700 mg/kg morphine and 0 2 mg/kg neostigmine simultaneously the mean time of survival was 18 minutes (14–24 minutes) Morphine was found in the brain at an average concentration of 27 µg/g (table 1 column 3) For comparison, 12 rats were given 700 mg/kg morphine only and were killed at times similar to the times of death in the above mentioned group with an average survival time of 19 minutes The mean concentration of morphine found in the brains was 24 7 µg/g (table 1, column 4), which did not significantly differ from what was found in the group receiving both morphine and neostigmine

Discussion

In the experiments reported here, neostigmine was found not to influence the amounts of morphine in the brains of rats, when the drugs were given either intravenously or intraperitoneally (compare Methodo-

logical Considerations and table 1) Our results are in agreement with those of SZERB & MCCURDY (1956), who administered morphine intra venously to rats and determined morphine in the blood and in the brain Some of the rats were pretreated by subcutaneous administration of neostigmine, but no significant difference was found between these and the controls The hypothesis of KNOLL, KOMLÓS & TARDOS (1953) that cholinergic drugs increase the amounts of centrally acting analgesics in the brain is, therefore, invalid

According to unpublished results of our own, it appears that morphine is found almost exclusively as "free" morphine in the brain, whether given alone or with neostigmine This indicates that our results are not consistent with any suppressed inactivation of morphine and pethidine by neostig mine in the liver, as was suggested by KOMLÓS & KOMLOS SZASZ (1954) At any rate, our results imply that the potentiating action of neostigmine on morphine analgaesia and on the lethal effect of morphine must be conditioned in some other way than by increasing the amounts of mor phine in the brain

Morphine exerts a central depressant effect on respiration, whereas neostigmine affects the respiration peripherally (SCHAUMANN 1958) In our experiments all the rats injected with both morphine and neostigmine died, whereas all the rats injected with morphine or neostigmine separetely survived Death was characterized by flaccid respiratory arrest KOMLÓS PÓRSZASZ & KNOLL (1950) found that neostigmine increased the respira tory depression induced by morphine in young rabbits Thus the deaths may probably have been due to a combined central and peripheral de pression of respiration, exerted by morphine and neostigmine respec tively

Simultaneous intraperitoneal administration of morphine and neo stigmine may lead to formation of one or more substances in the or ganism that can introduce an analytical error into the polarographic determination of morphine When however paper chromatography was included in the analytical procedure, this error was eliminated (fig 1) It is remarkable that this error never occurred during analysis of the brains of morphine tolerant rats (fig 1) It appears as though the tolerant rats are unable to produce the substance or substances that may interfere with the polarographic determination of morphine in the brains of non-tolerant rats that have been injected with morphine and neostigmine intraperitoneally

The polarographic method used depends on the presence of free phenol groups without substituents in the ortho position It is, therefore possible that the interfering substances may be phenol derivatives, but no further attempts have been made to isolate or to identify these substances

Summary

Morphine and neostigmine were given intraperitoneally to morphine tolerant and non tolerant male albino rats Morphine and neostigmine given together proved lethal to all rats For comparison, the rats given morphine alone were killed at approximately the same times as those at which the rats died from the injections of morphine and neostigmine

Morphine was determined in the brains of rats in both groups Neostigmine was not found to increase the amounts of morphine in the brain

The simultaneous intraperitoneal administration of morphine and neostigmine can lead to erroneous determinations of morphine However this was overcome, when, paper chromatography was included in the analytical procedure, and this error was not met with during analysis of the brains of the tolerant rats

Acknowledgements

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The Influence of Glucocorticosteroids and Corticotropic Hormone on Output of Human Urinary Trypsin Inhibitor (and Hyaluronidase Inhibitor)

By

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(Received July 7, 1962)

The urinary trypsin inhibitor (TI) has been known for many years It also inhibits chymotrypsin, plasmin (SCHULMAN 1955, ASTRUP *et al* 1959) and the non proteolytic enzyme hyaluronidase (FAARVANG & KNUDSEN 1962) It has been suggested that compounds in the organism that inhibit proteases or hyaluronidase may act as antagonists in the enzymatic mechanisms affecting the pathogenesis of anaphylaxis (UNGAR 1953 UNGAR & DAMGAARD 1954, HERBERTS 1955) and rheumatic diseases (for review see DORFMAN & MATTHEWS 1955) Since the urinary TI apparently possesses both of these properties, it is important to elucidate its behavior in the organism

The amount of TI excreted varies widely from one normal person to another (DILLARD 1950, FAARVANG 1957 & 1959a, MAYEHIRO 1959), ranging from 0 to 30 000 units per 24 hour (FAARVANG 1959a) However, in each individual the day to day variation as measured over 3 consecutive days is much smaller (0-50% average ±32% of the mean) and independent of the magnitude of the output From time to time an individual can, however, adjust his 24-hour output to higher or lower levels (FAARVANG 1962) The hourly excretion during the day often varies considerably (DILLARD 1950, KORSGAARD CHRISTENSEN 1958, FAARVANG 1958 & 1959a)

Different conditions, such as acute and chronic infectious diseases, artificially induced fever, injections of gold compounds, surgical intervention, coronary thrombosis and pregnancy, increase the output of TI (MÜLLER 1908 BAUER & REICH 1909, DOBLIN 1910, DILLARD 1950, FAARVANG 1957 & 1959b, MAYEHIRO 1959 & 1960) On the assumption that stress, a common factor in most of these varied conditions, could cause

the increase, the effects of adrenocorticotropic hormone, cortisol and cortisone were studied in a few subjects (FAARVANG 1957 & 1958) The results obtained showed that these hormones increased the output of the TI That an intimate relationship exists between the concentration of these hormones in the organism and the TI output was also suggested by the finding that the hourly outputs of TI and of 17-ketogenic steroids and 17 ketosteroids nearly parallel each other during the day and that the 24 hour output of the TI during normal pregnancy follows the variations in plasma concentration of 17-hydroxycorticosteroids (FAARVANG 1958 1959a & 1959b)

The activity in the organism of the unknown system that determines the output of TI could depend directly and exclusively on the concentration of cortisol (or other glucocorticosteroids) present However, no correlation between the 24 hour output of TI and the 24 hour production of cortisol (as measured by the 24 hour output of 17 ketogenic steroids) was found, suggesting that additional factors are involved in regulating the activity of this system (FAARVANG 1959a & 1962)

The purpose of the study reported here was to extend the earlier observations on the effect of adrenocorticotropic hormone and of different compounds with glucocorticosteroid activities on the output of urinary TI in order to establish this as a function of the glucocorticosteroids and especially to study quantitative individual variations in response to a given amount of hormone

Material and Methods

The investigations were carried out on 28 females and 24 males between the ages of 20 and 60 years The material comprised two groups one consisting of apparently normal persons who lived at home during the period of examination and a group of patients hospitalised for minor diseases (mild cases of neuroses or constipation without any signs of organic disorders and persons with mild cases of lumbago sciatica without symptoms of other diseases) Their body weights ranged from 50 to 80 kg

The 24 hour outputs of TI were measured for 5 consecutive days in each of the subjects The average value (*control level*) of the 24 hour excretions (*control values*) in [illegible]

[illegible] d

remain relatively constant during the last two days of examination [illegible] s in the TI output during these two days were considered an effect of the hormone [illegible] hormone 100 mg cortisol 100 mg

[illegible]

Below the excretions of TI during these days are [illegible] *tion value* and the increases are termed *first and second day increase*

The adrenocorticotropic hormone was given intramuscularly in a single daily dose at the beginning of the 24 hour period (between 7 and 9 a m) Cortisol was given

orally in 5 daily doses of 20 mg each, cortisone and prednisone were given 4 times daily in doses of 25 and 5 mg respectively The first dose of these hormones was given at the beg nning of the 24-hour period The 4 consecutive doses of cortisol were administrated at 3 hourly intervals, the 3 consecutive doses of cortisone acetate and prednisone were administrated at 4-hourly intervals The last dose of the day was given 12 hours before the end of each 24-hour period Triamcinolone was distributed over 4 da ly doses which were given at the same times as the cortisone or prednisolone

The concentrations of TI in the urine samples were determined by the inhibition of the proteolytic activity of crystalline trypsin containing 24 4 Anson units per g (kindly supplied by the *Novo Laboratories* Copenhagen), with casein as substrate, and determining the degree of digestion by the biuret method (for details see Faarvang 1959a) The urine specimens were kept at room temperature in 1 5 1 bottles containing 5 ml of chloroform during the collection period and then stored at −8 C until analysed A few specimens containing small amounts of glucose were dialysed through cellophane against isotonic sodium chloride before the measurements were made (Faarvang 1959a) All samples were adjusted to pH 7 to 8 with NaOH or HCl None of the specimens showed a positive Hellers test for protein

Results

1 *Effect of Adrenocorticotropic Hormone*

Control values control levels and first and second day of stimulation values are shown in table 1

The control values range from 0 to about 10 0 × 10^3 units In accordance with our earlier findings (FAARVANG 1962), an individual s TI output varies only slightly from day to day during the control period The control levels range from 0 to about 8 0 × 10^3 units During hormone stimulation the output increased in all subjects The values were highest

Table 1

Effect of 25 i u ACTH on output of urinary trypsin inhibitor
Values in 10^3 units

Name	Sex	Control values 1st	Control values 2nd	Control values 3rd	Day of stimulation values 1st	Day of stimulation values 2nd	Control level	Second day increase
G P	F	0	0	0	1 3	1 4	0	1 4
B S	F	0	0	0 4	3 2	7 5	0 1	7 4
H F	M	0 9	1 1	1 4	2 0	2 4	1 1	1 3
P M H	M	1 3	0 9	0 0	1 5	4 0	0 7	3 3
G J	F	2 0	1 2	0 9	4 1	4 7	1 4	3 3
R B	F	1 6	2 1	1 9	7 8	8 0	1 9	6 1
T C P	M	3 1	2 9	1 5	5 1	11 8	2 5	9 3
V F	F	4 3	4 0	8 1	13 7	16 2	5 3	10 9
T P	M	7 0	5 1	5 7	16 2	29 0	5 9	23 1
M S	F	10 5	6 3	7 3	15 4	20 7	7 9	12.8

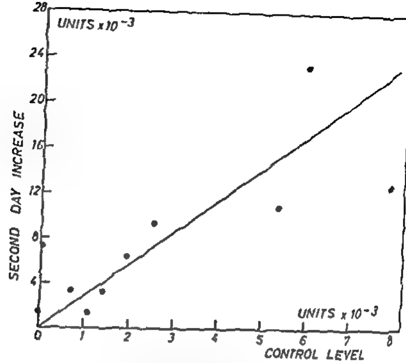

Fig 1 Relationship between control levels and second day increases in ACTH stimulated persons
Abscissa Control level in 10^3 units
Ordinate Second day increase in 10^3 units

in the second day increases, which range from 1 4 × 10^3 to 23 1 × 10^3 units The second day increase rose with rising control level, as seen in fig 1

The points are distributed around a line of direct proportionality Statistical analysis shows a significant positive correlation between control levels and second day increases The coefficient of correlation is +0 76 ($p < 0\ 05$)

2 *The Effect of 100 mg Cortisol*

The effect of 100 mg of exogenous cortisol on the TI output was studied in 5 subjects One of these persons (G P) also participated in the study of adrenocorticotropic hormone

As seen in table 2, the control values ranged from 0 to 6 0 × 10^3 units with only small variations in the individual Control levels ranged from 0 to about 6 0 × 10^3 units All the subjects increased their outputs during stimulation The second day increases were again considerably larger than those on the first day of stimulation, and a relationship was again found between the varying second day increases and the control levels

As seen in fig 2, the points are again distributed around a line of direct proportionality This suggests that, as in the persons stimulated by adreno

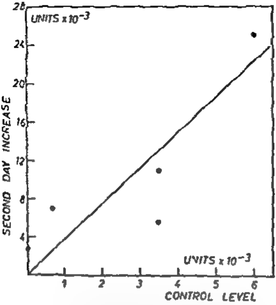

Fig 2 Relationship between control levels and second day increases in cortisol stimulated persons *Abscissa* and *ordinate* as in Fig 1

Table 2

Effect of 100 mg cortisol on output of urinary trypsin inhibitor
Values in 10^3 units

Name	Sex	Control values			Day of stimulation values		Control level	Second day increase
		1st	2nd	3rd	1st	2nd		
G P	F	0	0	0	21	28	0	28
E E	F	06	06	09	49	78	07	71
W A	M	29	43	32	56	92	35	57
H L	M	37	41	26	57	145	35	110
I M	F	56	58	66	169	310	60	250

corticotropic hormone, there is a relation between the second day increases and the magnitude of the control levels This relation tends to be approximately a direct proportionality

3 *Effect of Cortisone Acetate*

This phase of the investigation was carried out on 42 persons, four of whom had participated in the preceeding experiments (G P , H.F , V F , and T P) The results are shown in table 3

Table 3

Effect of 100 mg of cortisone acetate on output of urinary trypsin inhibitor
Values in 10^3 units

Name	Sex	Control values			Day of stimulation values		Control level	Second day in crease	Sec day of stim val / Control level
		1st	2nd	3rd	1st	2nd			
M L	F	0	0	0	0	0 8	0	0 8	–
G E N	F	0	0	0	0	0	0	0	–
G P	F	0	0	0	0	3 1	0	3 1	–
H J F	M	0 8	0	0	0	0 9	0 3	0 6	3 00
G J	F	0 5	0 4	0 6	3 2	3 2	0 5	2 7	6 40
H J	F	0	0 9	0 9	3 5	3 7	0 6	3 1	6 17
F H	F	1 5	0 7	0	2 0	2 7	0 7	2 0	3 86
F M	F	0 8	1 4	0	0	1 6	0 7	0 9	2.29
D I	F	1 5	0 6	0 4	3 3	2 1	0 8	1 3	2 63
S A J	M	1 2	1 0	0 7	1 7	3 0	1 0	2 0	3 00
H F	M	1 9	0 5	0 8	1 8	0 9	1 1	0 2	0 82
A H	F	1 9	0 7	1 1	2 3	2 2	1 2	1 0	1 83
Bo J	M	1 4	1 3	1 5	1 8	1 5	1 4	0 1	1 07
R	M	1 9	1 1	0 7	2 0	1 6	1 2	0 4	1 33
H L	M	2 4	1 1	1 7	1 4	6 9	1 7	5 2	4 06
L N	M	1 5	1 8	1 8	5 5	4 4	1 7	2 7	2 59
O P	F	1 4	2 6	1 3	3 6	6 0	1 8	4 2	3 33
L J	F	2 4	2 1	1 7	5 1	7 5	2 1	5 4	3 57
I B	F	2 9	2 2	1 4	2 6	5 4	2 2	3 2	2 45
J L	M	1 7	1 9	2 9	4 4	5 2	2 2	3 0	2 36
R A	F	2 7	3 0	2 6	5 6	6 5	2 8	3 7	2 32
L	M	3 8	3 2	2 0	2 3	5 6	3 0	2 6	1 87
E F	F	2 9	2 9	3 3	6 1	5 2	3 0	2 2	1 73
E A	F	4 2	2 8	2 7	3 9	4 7	3 2	1 5	1 44
A B	F	4 2	2 5	2 8	6 2	11 2	3 2	8 0	3 50
R J	M	3 3	2 6	3 3	3 3	14 5	3 2	11 3	4 53
S H H	M	2 7	3 7	3 8	6 8	8 5	3 4	5 1	2 50
I M J	F	3 4	3 5	4 5	8 3	12 0	3 8	8 2	3 16
K J	F	4 0	2 8	5 0	10 7	10 4	3 9	6 5	2 67
S A	F	3 8	3 6	5 1	9 6	13 2	4 2	9 0	3 14
T P	M	5 2	4 3	4 2	7 1	6 1	4 6	1 5	1 33
B L	F	4 6	4 6	6 7	12 3	18 7	5 3	3 4	1 64
P K	M	4 9	3 8	7 6	13 7	12 1	5 4	6 7	2 24
O L	M	3 9	5 9	6 6	7 9	8 6	5 5	3 1	1 56
R B L	M	4 8	5 1	8 4	7 1	5 5	6 1	0 6	0 90
V F	F	7 5	7 2	7 2	13 6	21 0	7 3	13 7	2 88
J C N	M	7 3	9 9	9 4	14 0	14 9	8 9	6 0	1 67
Bo B	M	10 3	9 0	12 5	15 8	13 0	10 6	2 4	1 23
A S C	M	13 9	12 5	7 1	15 8	10 6	11 2	−0 6	0 95
Be B	F	16 6	26 0	23 0	41 5	32 2	21 9	10 3	1 47
P J	M	30 2	30 0	16 6	18 8	27 2	28 3	−1 1	0 96
H K	M		36 0	30 1	41 2	43 6	33 1	10 5	1 32

Control values ranged here from 0 to 36 0 × 10^3 units and thus covered the total range previously recorded for the normal spontaneous 24 hour output Control levels ranged from 0 to about 30 0 × 10^3 units Probit diagrams show a logarithmic normal distribution of both control levels

and first and second day of stimulation values, logarithms of the values were consequently used to calculate the following average values

In most of the subjects cortisone administration caused an increased TI excretion, but in a few no increases were observed This occurred in persons with small or large control levels The second day increases for many of the subjects whose TI outputs increased with stimulation were again larger than those on the first day of stimulation

The average of the second day of stimulation values was 6.1×10^3 units ($\log_{10} = 3.786$ with a standard deviation of 0.402), and the average of the control levels 2.9×10^3 units ($\log_{10} = 3.457$ with a standard deviation of 0.463) The average second day increase was 3.2×10^3 units, and this difference is statistically significant ($p < 0.001$) As to co variation between control levels and the second day increases, in principle the same relation was found as in the previous parts of the investigation There is a statistically significant, though rather low, correlation between the values The coefficient of correlation = +0.33 ($p < 0.05$)

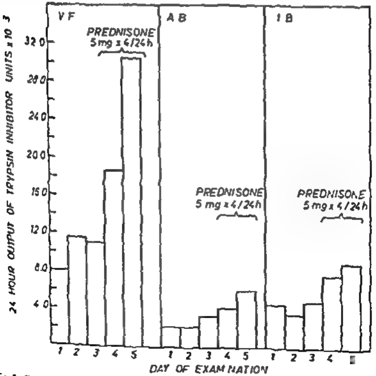

Fig. 3 Effect of prednisone on output of urinary trypsin inhibitor
Abscissa Day of examination
Ordinate Trypsin inhibitor output in 10^3 units/24 hours.

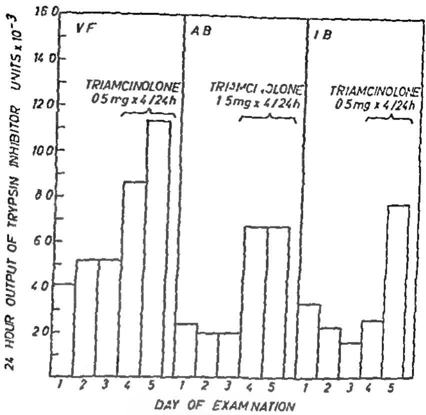

Fig 4 Effects of triamcinolone on output of urinary trypsin inhibitor
Abscissa and *ordinate* as in Fig 3

4 *Effect of Prednisone and Triamcinolone*

The effects of prednisone and triamcinolone were studied in the 3 subjects I B , A B and V F , who also participated in one or more of the previous parts of the investigation

The dosage of prednisone, 20 mg per day, was distributed in 4 daily doses As seen in fig 3, prednisone produced increased TI output in all 3 subjects

The dosage of triamcinolone used ranged from 2 to 6 mg per day, and was also distributed in 4 daily doses It also produced increased TI outputs in all the subjects (Fig 4)

Discussion and Conclusions

The results of the investigation confirm and extend the findings of the preliminary studies (FAARVANG 1957 & 1958) The urinary TI output increased in all subjects treated with adrenocorticotropic hormone or cortisol, and with cortisone the average second day increase was statistically significant

It is well-known that cortisone and cortisol have to some extent,

beside their effects as glucocorticosteroid, an effect on electrolyte metabolism qualitatively similar to that of the mineralocorticosteroids, aldosterone and deoxycorticosterone It is unlikely that the effect of cortisol and cortisone on TI output is connected with this activity of the hormones The effect of aldosterone and desoxycorticosterone on the TI output has not been studied, but prednisone, in the doses employed, exhibit only a small mineralocorticosteroid effect, if any, and the doses of triamcinolone used does not show this (FREYBERG *et al* 1958), though they had a considerable effect on TI output It can therefore be concluded that the urinary TI output is a function of the glucocorticosteroids and that an increase of the concentration of these hormones or of compounds with glucocorticosteroid activity tends to produce an increased output of the inhibitor Our earlier finding, that the hourly TI output during the day in an individual follows the cortisol production, indicates that this conclusion is not only valid for increases in the hormone concentration produced by pharmacological doses of the hormones, but also for increases produced by physiological changes in endogenous cortisol production

In many of the subjects treated with cortisol or cortisone, the second day increases were considerably larger than those on the first day of stimulation In a few persons we followed the 24-hour output of TI for more than two days (3 to 6 days) during continued treatment with corticosteroids at the same dosage as those employed in the main study Whereas relatively large differences were observed between the outputs of the first and second day of treatment, only small differences were observed in the individual between the second day and subsequent daily output This inertness in the adaption to higher concentrations of glucocorticoid apparently manifests itself predominantly towards unphysiological increases in concentration, since, as already mentioned, the spontaneous output in the individual can follow the physiological changes in the cortisol production during the day

In persons stimulated by adrenocorticotropic hormone, cortisol or cortisone the same amount of the single hormone produced different increases on the second day of stimulation although the maximal response to the excessive amount of hormone was apparently attained on this day Since the level of *spontaneous TI excretion in the same individual* remains relatively constant from day to day (*vide supra*), the second day increases must have been caused by the excess amount of glucocorticosteroid affecting the subjects

Concerning the cause of the variations from person to person observed in the second day increases in the cortisol subjects, one might regard variations *in* excess amount of cortisol as a possible explanation However, it can indirectly be shown that this cannot be the only cause

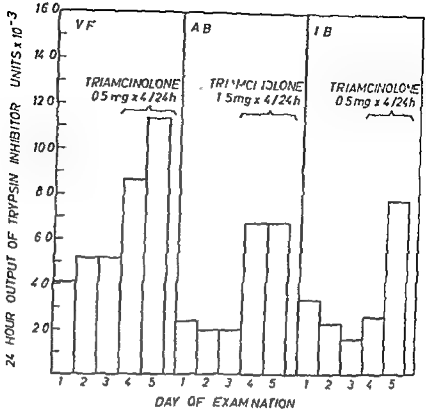

Fig 4 Effects of triamcinolone on output of urinary trypsin inhibitor *Abscissa* and *ordinate* as in Fig 3

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The excess amount of cortisol is the difference between the amount of cortisol affecting the person on the second day of stimulation and the amount influencing him in the control period The amount affecting the person on the second day of stimulation was nearly the same in all the persons, since the glucocorticosteroid dosage employed depresses the adrenal function considerably in normal persons (LIDDLE 1960) and since a fairly constant percentage of orally administrated cortisol is absorbed from the intestines (COPE & BLACK 1959) The excess amount therefore varies inversely with the amount of cortisol produced in the control period If this *production is great (which would in itself tend to produce a* large control level of TI), the excess amount is small, and one would therefore expect a small second day increase by large control levels if variations in the excess amount were the primary cause of the variations in the second day increases On the contrary, we find a positive correlation between control levels and second day increases

Other factors must therefore be acting As the second day increase is caused by the *excess amount of cortisol, these factors must act* by determining *the output of TI produced by a given amount of glucocorticosteroid*, according to generally employed terminology in biology, they act by determining the person's *sensitivity* to this function of cortisol

The assumption that the sensitivity to cortisol (endogenous as well as exogenous) is a fairly constant factor in the individual from day to day during the period of examination, but varies from person to person, gives a simple explanation of the positive correlation between control levels and second day increases

It seems evident that individual variation in the TI output produced by a unit quantity of cortisol in the precense of a relatively constant sensitivity in the individual can also explain the correlation between control levels and second day increases found in the subjects stimulated by adrenocorticotropic hormone, when assuming that the sensitivity to cortisol and cortisone co vary from person to person, the same considerations can be applied to the results obtained on the cortisone stimulated persons

The simplest explanation of the results obtained seems therefore to be that a person's urinary TI output is determined by at least two variables namely by the amount of glucocorticosteroid affecting him and by his individual sensitivity to this function of the hormone It seems probable that this sensitivity must be distinguished from the initial inertia of the TI output to excessive concentrations of glucocorticosteroids which was observed in the individual subject, but the factors that determine these phenomena are not

they are bound up

system in the organism that produces TI

Summary

1 Output of the urinary trypsin inhibitor is controlled by the glucocorticosteroids

2 Increased concentration of the hormones in the organism tends to produce increased output of the inhibitor

3 The sensitivity of the function to a given glucocorticosteroid shows large individual variations

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Acta pharmacol et toxicol 1962, 19, 305–311

From the Biological Institute of the Carlsberg Foundation and the Medical Department A the University Hospital, Copenhagen

Studies on the Quantitative Relationship between Cortisol Production and Spontaneous Output of Urinary Trypsin Inhibitor in Man

By

Hans J. Faarvang

(Received July 7 1962)

In the preceeding communication it was found that the output of urinary trypsin inhibitor (TI) is determined by at least two variables (1) the amount of glucocorticosteroid affecting the organism and (2) the individual's sensitivity to the hormone in relation to this function, which was defined as the TI output produced by 1 mg of the hormone in question

If the total output of urinary TI is produced by the glucocorticoids, and on the assumption that an individual's sensitivity does not change with alterations in the amount of glucocorticosteroid, the output is characterised by the equation

$$\text{TI} = \text{Amount of glucocorticoids (in mg)} \times \text{Sensitivity to the hormone} \tag{1}$$

and an individual s spontaneous 24-hour output level of the inhibitor (control level ') by

$$\text{Control level} = \text{Average 24 hour cortisol production (in mg)} \times \text{Sensitivity to cortisol} \tag{2}$$

It was, moreover, suggested that the sensitivities to cortisol and to cortisone vary together from person to person If this is so,

$$\text{Sensitivity to cortisol} = \text{Sensitivity to cortisone} \times k \tag{3}$$

where k is a constant common to different individuals Thus (2) can be converted to

$$\text{Control level} = \text{Average 24 hour cortisol production (in mg)} \times \text{Sensitivity to cortisone} \times k \tag{4}$$

or

$$\frac{\text{Control level}}{\text{sensitivity to cortisone}} = \text{Average 24-hour cortisol production (in mg)} \times k \tag{5}$$

Shulmau, N R.: A proteolytic inhibitor with anticoagulant activity separated from urine and plasma. *J. Biol Chem* 1955, 213, 655–671.
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If the premises for equation (5), i e the equations (1) to (4), are valid, a direct proportionality must be present between the ratio $\frac{\text{Control level}}{\text{Sensitivity cortisone}}$ and the average 24-hour production of cortisol in the control period

In this communication it will be shown, that this proportionality exists, in a group of randomly selected normal subjects

Material and Methods

The TI outputs from 17 of the subjects in the group of cortisone stimulated subjects described in the earlier paper were used in our study In all these subjects the 24 hour outputs of 17-ketogenic steroids (17 KGS) had been determined for 2 or 3 days of the control period In the following the individual average values of these outputs will be used as relative measures of the subjects' average cortisol production in the control period It seems justifiable to do this since it has been shown that there is a positive correlation between the urinary output of 17-KGS and that of the Porter Silber chromogens (MONHAM & NABARRO 1956) and since it appears from results obtained by PETERSON (1959) that there is a direct proportionality between the urinary output of Porter Silber chromogens and the production of cortisol as measured by the urinary metabolite specific activity method The amounts of 17 KGS in the urine specimens were measured by the Hormone Department of the Statens Serum Institut, Copenhagen (for method see JORGENSEN 1957)

As described in the preceeding paper 100 mg of cortisone acetate were given orally to each subject It will also be assumed here that the subject's optimal TI output responsiveness to this dose of the hormone was attained on the second day of stimulation It is further assumed that the dose of cortisone acetate produces a total inhibition of endogenous cortisol production and that the percentage of cortisone acetate absorbed is fairly constant from person to person

If equation (1) is valid the total TI output on the second day of stimulation is therefore produced by the exogenic cortisone acetate and thus

$$\text{Second day of stimulation value/100 (i e the TI output produced by 1 mg of orally administrated cortisone acetate)} = k_1 \times \text{Sensitivity to cortisone} \qquad (6)$$

where k_1 is a constant common for all the subjects The second day of stimulation value/100 can then be used as a *relative* measure of the subject s sensitivity to cortisone

(Further details of material methods and terminology can be found in the preceeding paper)

Results

The subjects' control levels, the second day of stimulation values /100, the ratios $\frac{\text{Control level}}{\text{Second day of stimulation value /100}}$ and the individual and average 24-hour outputs of 17-KGS are shown in table 1 Individual TI values will be found in the previous paper

Table 1

Name	Sex	Control level (units)	2nd day of stim val /100 (units)	Control level / 2nd day of st m val 100	17 KGS in mg 24hours of control period			
					1st day	2nd day	3rd day	average
FH	F	0 7 × 10^3	27	26	5 8	4 8	3 9	4 8
Bo J	M	1 4 × –	15	93	12 8	9 9	8 8	10 5
R	M	1 2 × –	16	75	14 6	13 7	13 4	13 9
I B	F	2 2 × –	54	41	6 5	6 2	6 4	6 4
J L	M	2 2 × –	52	42	4 9	3 1	6 3	4 8
L	M	3 0 × –	56	54	5 6	8 3	6 8	6 9
E F	F	3 0 × –	52	58	5 6	5 1	11 8	7 5
A B	F	3 2 × –	112	29	5 6	7 0	5 5	6 0
S H H	M	3 4 × –	85	40	8 4	7 6	8 5	8 2
I M J	F	3 8 × –	120	32	7 5	–	8 3	7 9
K J	F	3 9 × –	104	38	6 4	4 3	3 3	4 7
T P	M	4 6 × –	61	75	15 3	12 8	14 4	14 2
O L	F	5 5 × –	86	64	–	5 7	9 7	7 5
R B L	M	6 1 × –	55	111	17 5	13 4	8 4	13 1
V F	F	7 3 × –	210	35	5 7	7 9	4 7	6 1
Bo B	M	10 6 × –	130	82	15 7	11 5	14 0	13 7
P J	M	28 3 × –	272	104	10 1	11 1	12 2	11 1

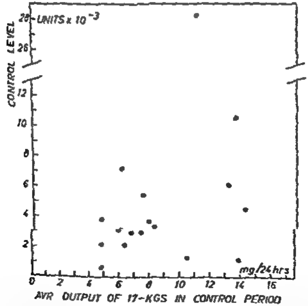

Fig 1 Absence of correlation between control level and average 24-hour output of 17 ketogenic steroids in the control period
Absc ssa Average 24-hour output of 17 ketogenic steroids in the control period
Ordinate Control level in 10^3 units

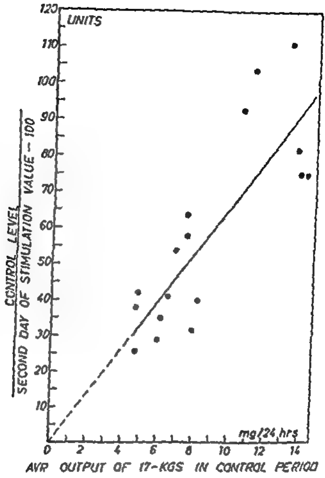

Fig 2 Correlation between the ratio $\frac{\text{Control level}}{\text{Second day of stim value/100}}$ and the average 24 hour output of 17 ketogenic steroids in the control period

Abscissa Average 24 hour output of 17 ketogenic steroids in the control period

Ordinate $\frac{\text{Control level}}{\text{Second day of stimulation value 100}}$

The straight line is fitted through points calculated from equation (7)

In fig 1 the control levels are related to the corresponding average 24-hour outputs of 17-KGS The points appear to be distributed fortuitously, a statistical analysis indeed shows that there is no correlation between the two variables, the coefficient of correlation being $+0\ 36$ ($0\ 20 > p > 0\ 10$)

In fig 2 the ratios $\frac{\text{Control level}}{\text{second day of stimulation value/100}}$ are related to the same average 24-hour outputs of 17-KGS It appears directly that the two variables are correlated, apparently as a direct proportionality and with no deflection even for the largest 17-KGS outputs In accordance the high correlation coefficient, $+0\ 81$ was found ($p < 0\ 001$)

The regression line characterising the quantitative relationship between the two variables is given by the equation

$$\frac{\text{Control level}}{\text{Second day of stim val /100}} = 6\,6 \times 17\ \text{KGS} \tag{7}$$

In the statistical development of (7) the logarithms of the variables have been employed so that the variables and proportions between them become normally distributed

Writing (7) in expanded form

$$\frac{\text{Control level}}{\text{second day of stim val /100}} = a \times (17\ \text{KGS})^{b} \tag{8}$$

where the parameter a is the factor of proportionality in (7) and b is introduced to re examine the hypothesis of direct proportionality If now $b = 1$, the hypothesis is true If $b > 1$, the ratio

$$\frac{\text{Control level}}{\text{second day of stim val /100}}$$

increases more than proportionally to 17-KGS and if $b < 1$, the ratio increases more slowly

By common regression analysis we find that $b = 0\,97 \pm 0\,18$ Consequently, b does not deviate significantly from 1 and the results obtained are hence not inconsistent with the hypothesis of direct proportionality

For the parameter a we find $\log a = 0\,817 \pm 0\,028$ giving $a = 6\,6$ with a standard error of about 0 4

The 17 subjects' deviations from the strict proportionality are characterised by the logarithmic standard deviation $s = 0\,118$, corresponding to a mean deviation of about 30% in absolute values

Discussion and Conclusions

In the preceding paper it was concluded that the TI output is determined by at least two variables, namely the amount of glucocorticoid affecting the organism and the sensitivity to the hormone in relation to this specific function It seems justifiable from the results presented here to conclude that the variations in spontaneous TI output in urine are determined exclusively by variations in the two variables

It is now understandable why no correlation could be found between the normal TI output and the cortisol production in different normal persons (FAARVANG 1959), although there seemed to be a close interrelationship between the two functions According to the findings reported here, the total TI output in urine depends on the glucocorticoids, and an increase in the amount of glucocorticoid affecting the organism

tends to produce a proportional increase in the TI output However, the TI output produced by a unit weight of the hormone changes from person to person, and this latter variation conceals the existing relationship

In fact, it also appears from the results that a greater part of the person-to-person variation in control levels is caused by variations in sensitivity than by variations in amount of endogenous cortisol The extreme limits of the average 24 hour outputs of 17-KGS are 4 7 and 14 2 mg, giving a maximal variation in cortisol productions of about 3 times, whereas the extreme limits of the second day of stimulation value/100, which gives a relative measure of the subjects' sensitivities to cortisol, are 15 and 272 units, giving a maximal variation in sensitivity of about 18 times

Moreover, it seems evident that the sensitivity to cortisol (and to cortisone) must have been fairly constant from day to day within the total 5 day period in the individual subject, since it is a necessary condition for the results obtained that the sensitivity to cortisone estimated in the 5th day can be employed as a relative measure of the sensitivity to cortisol in the first 3 days of the period That a certain variation has occurred can, however, not be excluded This variation may, together with analytical errors, errors in urine collection, differences in intestinal absorption of cortisone acetate and errors introduced by employing the output of 17-KGS as a measure of the cortisol production, be contributing factors to the observed spreading of the points from the strict proportionality, as shown in fig 2

Summary

In an earlier study it has been shown that the output of urinary trypsin inhibitor is influenced by the glucocorticoids and that the sensitivity to the hormones in relation to this function shows individual variations

The results presented here suggest that the total spontaneous TI output in man is produced by cortisol An increase in cortisol production increases the output, but individual variations in sensitivity to the hormone determine to an even greater extent the individual variations in output

Cortisone has the same effect on the output of trypsin inhibitor as cortisol and the sensitivities to cortisol and cortisone covary from person to person

Acknowledgements

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Research Foundation, the Foundation of the "Leo" Chemical Factory, the Michaelsen Foundation and the P Carl Petersens Foundation Miss Ingrid Wirth and my wife have assisted meticulously by the technical work

The statistical calculations were made in cooperation with cand act M Nyholm I/S Statistica, Copenhagen

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Acta pharmacol et toxicol 1962, 19, 312–324

From the Department of Forensic Medicine, University of Edinburgh

The Rapid Separation of Drugs and Poisons by High Temperature Reversed Phase Paper Chromatography.*)
2. Phenothiazine Tranquillizers and Imipramine

By

Harold V. Street.

(Received May 14 1962)

Several workers have applied conventional methods of chromatography to separating phenothiazine derivatives In their chapter on alkaloids, JACKSON & MOSS (1960) describe the use of four different solvent systems, of which they state that two are of little value for the phenothiazines, with the other two solvents, the phenothiazines form long elongated spots FARMILO & GENEST (1961) also refer to several phenothiazines in their chapter on alkaloids They list four different solvent systems for pheno thiazine separations TABAU & VIGNE (1958) describe a solvent for descending chromatography of N-substituted phenothiazines, but they did not obtain good resolution, for example, the R_f values of 8 pheno thiazine derivatives were 0 80, 0 83, 0 85, 0 86, 0 87, 0 88, 0 89 and 0 91

*Recent articles, (*STREET *1962 a, b, c), have described experiments in* which some of the derivatives of barbituric acid were separated with aqueous solvents by paper-chromatography on ester-impregnated filter-paper

The outstanding feature of these experiments was that increasing the temperature at which chromatography was carried out greatly increased the speed of resolution of the applied compounds Because *aqueous* solvents were employed, it was possible to use temperatures up to 86°C Indeed, we describe here experiments in which chromatography is carried out at 95° This type of chromatography is probably nearer to true partition chromatography than most other forms of paper-chromatography The R_f values *of the compounds being separated will depend*

*) Rapid separation of drugs and poisons by high temperature reversed phase paper chromatography (1) Street (1962 b)

largely on their partition coefficients between the ester with which the paper is impregnated and the solvent used It is important to note that the partition coefficients may vary with temperature There are, therefore, five variables that can be controlled and used to effect resolution These variables are (1) type of ester on the paper (2) amount of ester on the paper, (3) pH of the aqueous solvent, (4) concentration of the aqueous solvents and (5) temperature

Materials

Esters Used to Impregnate the Paper

The first requirement is that the boiling point of the ester shall be well above the temperature at which chromatography is carried out On the other hand, it is preferable to use an ester that is liquid at this temperature Over fifty esters have been investigated in connexion with the separation of the 5 5 disubstituted barbituric acid derivatives, apart from some minor differences in R_f values, the results have been similar for most of the esters The compound, so far, found most satisfactory is glycerol tributyrate (Tributyrin) This boils at 315°C (at 760 mm of Hg), when used as a 10% (v/v) solution in acetone, it gives excellent results not only with the barbiturates but with the phenothiazines the classical alkaloids, neutral compounds and certain other substances It may be perhaps superfluous to mention that the chosen ester must not be soluble in water, but it is rather surprising to find that a mono ester of glycerol, namely glycerol mono ricinoleate, has been found useful for separating several mixtures of substances As the length of the carbon chain is reduced, the water solubility naturally increases, we find for example, that glycerol mono acetate which is freely soluble in water, is unsatisfactory for reversed phase work

Amount of Ester on the Paper

The amount of ester put on the paper is a compromise, obtained by using a 10 per cent solution Although 2 per cent solutions give slightly increased differences in R_f values between consecutive members of the series, considerable 'tailing' unfortunately is experienced At a concentration of 20%, the spots obtained are compact round and discrete, but all the R_f values are lowered and resolution is poor These observations emphasize the point that, in this type of chromatography, R_f values should not be taken as absolute but only as relative, and a standard must always be 'run' alongside the 'unknown' spot

pH and Concentration of the Aqueous Solvent

In the barbiturate separations, the relative R_f values of the separated spots do not vary appreciably with the pH of the solvent This is because the pK_1 values of the individual 5 5 disubstituted barbituric acid derivatives are similar and, according to BIGGS (1956), lie within the limits pH 7 6, to pH 8 6 We therefore find, as would be expected, that above pH 8 6, the barbiturates move more or less together as a group with an R_f value of about 0 8 or 0 9, whereas at pH 7 4, good resolution of the individual members of the group is obtained Even with a pH as low as 1 5, the resolution is still fairly good

Therefore, when we turn to the separation of a group of substances whose individual compounds have widely differing pK values, we find that we can produce striking differences in R_f values merely by altering the pH of the solvent The classical alkaloids lend themselves admirably to this type of treatment, but they are not considered further in this communication, which is concerned with the resolution of a number of phenothiazine derivatives, compounds also well suited to the reversed phase high temperature aqueous solvent technique It is perhaps pertinent to mention here that, as would be expected in true partition chromatography, the precise nature of the anions and cations used in the solvent appears to have little influence on the movement of the applied compounds Only the pH is important However, it is well to note that when "tailing" of the spots is experienced, it can sometimes be prevented by increasing the concentration of the buffer solution For example, ascending chromatography (at 86°C on filter paper impregnated with tributyrin) of the 5 5-disubstituted barbituric acid derivatives gives compact almost circular spots with an $^{M}/_{15}$ buffer solution (pH 7 4), whereas a more concentrated buffer solution (ca pH 4 5) is required to prevent "tailing" of the phenothiazines under similar chromatographic conditions

Much of the initial work with the phenothiazines was carried out with a 0 1 M solution of the tetracemin disodium (EDTA) This solution has a pH of about 4 5 It was subsequently found, that even more compact spots were obtained by increasing the buffer concentration A molar citrate buffer gives good results, but reduces the speed of solvent flow although the speed of solvent flow with a molar *acetate* buffer is little different from that with a 0 2 M – acetate buffer In general 0 2 M buffers give the best results Citrate buffers are more stable than acetate buffers at 95°C, but the colours with the Marquis reagent are more distinct with acetate rather than citrate on the paper No advantage is gained by using solvents whose concentration is greater than 1 M, indeed with them so much solute may be left on the paper as to interfere with the techniques used for locating the separated spots

Variation of the Temperature at which Chromatography is Carried out

In a previous communication, STREET (1962 b) has shown that the resolution of a mixture of barbiturates was improved by raising the temperature at which chromatography is carried out The temperatures used to illustrate this were 20°C, 62°C and 86°C Further, not only was the resolution improved but also the speed of obtaining this improved resolution was greatly increased, and without diffusion of the spots, which were thus compact and sharply defined

Experiments with aqueous solvents at 95°C have now produced even better resolution in a shorter time For some heat labile compounds, this temperature may be too high On the other hand, the short time of the 'run' may prevent *complete* destruction Digoxin, for example, has been successfully separated from unwanted material by chromatography for 15 minutes at 86°C

One cannot over emphasize the enormous difference in time required for resolution of the applied mixtures of compounds by this reversed-phase high temperature, aqueous solvent paper-chromatography, compared to the time required for conventional chromatography For example, the time required for chromatography of a number of nitrogenous compounds (including some phenothiazine derivatives) by the technique of JACKSON & MOSS (1960) is *17 hours*, it is seen from their diagram (page 402) that the length of some of the "spots" is almost half the length of the 'run' They themselves state that the phenothiazines give elongated spots by their technique It will be seen that the technique described herein (v infra) gives, by comparison, good resolution of a mixture of phenothiazines and a closely related compound imipramine (tofranil ®), in a matter of only 40 minutes and a useful but partial resolution in 20 minutes In an analytical toxicological search for the presence of a single phenothiazine, a 20-minute run may be sufficient Such a rapid separation will undoubtedly be of great value to the clinician, who may be confronted with a confused or comatosed patient suspected of taking drugs Further, the technique should be of value to those engaged in pharmacological studies of new drugs

Experimental

Preparation of the Paper

Initially, Whatman No 1 filter paper was used Experiments with Whatman Nos 3 3 MM, 4 and 50 have resulted in the general use of the No 3 grade This is a thick paper, even when wet it stands up well to the

For ascending chromatography, 6½ × 7 rectangular sheets of filter paper were dipped, as required, in a 10% (v/v) solution of glycerol tributyrate (tributyrin) in acetone blotted to remove excess liquid and then hung in the air for a few minutes to dry For horizontal circular chromatography, papers were cut as described by KAWERAU (1960)

Application of Phenothiazines to the Paper

The phenothiazines and imipramine were prepared as 1% (w/v) solutions in ethanol About 5 µl of each of these solutions were applied, by means of a fine glass capillary tube, as a spot at the line of origin on the ester impregnated paper The solvent was allowed to evaporate spontaneously

Buffers used as Solvents for Chromatography

The effects of pH and concentration of solvent on R_f values and on the shape of the spots have already been discussed The buffers used were prepared as described by VOGEL (1955)

Chromatography at Elevated Temperatures

For ascending chromatography, the tributyrin impregnated sheets of Whatman No 3 paper, containing the compounds to be separated, were folded into the shape of a cylinder, fastened with a paper clip and put into a beaker (containing the solvent) previously placed in a thermostatically controlled oven maintained at the required temperature The paper cylinder was put into the beaker without removing it from the oven A disc of plate glass, thickly smeared with silicone stopcock grease, served as a cover

For a cylinder of height 6½ inches a 20 minute 'run was used

Horizontal circular chromatography was carried out as described by KAWERAU (1960), the Whatman No 3 paper discs with slots were cut by hand with a pair of scissors before being dipped in tributyrin solution The flanges of the Kawerau apparatus (Supplied by the Shandon Scientific Co Ltd, London) and the paper were thickly smeared with silicone stopcock grease to prevent loss of water vapour For high temperature work it was found better to use a wick of rolled filter paper dipping into the solvent rather than the glass capillary supplied by the manufacturers As with the ascending technique, the apparatus was pre heated in an oven 'Running' times were either 20 or 40 minutes

Several experiments were carried out by both the ascending and the horizontal circular techniques in which a beaker of tributyrin was included in the chromatography chamber This produced no detectable difference in the final chromatograms

The temperatures at which chromatography was carried out were 62°, 86°, 90° or 95°C The 95°C 'runs' gave the best resolution

Location of the Separated Phenothiazines after Chromatography

After the chromatographic 'run' the wet papers were examined at 254 mμ (Hanovia "Chromatolite") and the position and colour of any fluorescent or absorbing spots were noted The papers were then dried with a hairdryer and re examined at 254 mμ Any change in colour of the spots was noted If several separate spots of the mixture to be separated have been placed on the paper the chromatogram may be cut into strips and each strip treated separately It is also possible to cut the *circular* chromatogram (from a single applied spot) into two or three thin strips and to treat each strip with a different locating reagent

Iodoplatinate is the first of such reagents into which the dried strip is dipped It reveals the phenothiazines (and imipramine) as dark blue-black areas on a reddish-brown background The other strips may be treated with the various concentrated sulphuric acid reagents, although the most information will be given by the modified Marquis reagent These reagents have had to be modified by shaking the sulphuric acid with solid anhydrous sodium sulphate, because the unmodified reagents react rapidly with the tributyrin on the paper and cause an almost immediate browning or blackening of the paper which makes detection of the spots impossible Addition of sodium sulphate to the acid delays the destruction of the tributyrin for about 10 minutes and so allows adequate time for noting the colours and positions of the separated spots or zones Unfortunately, even these modified strong acid reagents cannot be used on paper impregnated with glycerol monoricinoleate, because of immediate discolouration of the paper

Preparation of Location Reagents

Iodoplatinate reagent

Mix together 10 ml of 10% (w/v) aqueous platinic chloride solution and 90 ml of 10% (w/v) aqueous potassium iodide solution Dilute the mixture to 300 ml with water

Modified Sulphuric Acid Reagents

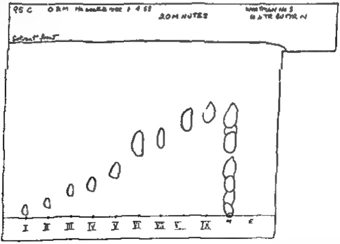
These reagents were prepared as described by Bamford (1951) except that in place of concentrated sulphuric acid, a mixture was used

Shake 200 ml of concentrated sulphuric acid with 50 g of anhydrous sodium sulphate for several minutes, and then allow the excess solid to settle and the mixture to cool

Results.

Mixtures of eight phenothiazines have been subjected to high temperature reversed-phase paper-chromatography – thiopropazate (I), trifluperazine (II), fluopromazine (III), thioridazine (IV), chlorpromazine (V), mepazine (VI), promethazine (VII) and promazine (VIII) A compound closely related to the phenothiazines, namely, imipramine (IX) was also included

Figure 1 shows the behaviour of compounds I to IX when subjected to 20 minutes ascending chromatography on tributyrin impregnated paper at pH 4 58 and 95°C The pattern shown in figure 2 was obtained on replacing the tributyrin by glycerol monoricinoleate, other conditions being as for figure 1 It will be noticed that the R_f values of all the compounds are lower on the ricinoleate paper Also the relative positions of fluopromazine and thioridazine are interchanged Figure 3 shows the results obtained by increasing the time to 40 minutes with the tributyrin impregnated paper



Trifluperazine (II)
e (VI) Prometha
impregnated with
ate reagent
20 mins

Solvent = 0 2M acetate bu

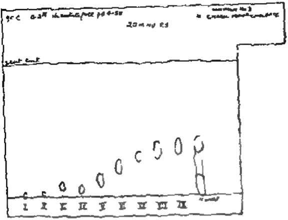


Fig 2 As for figure 1 except tributyrin replaced by glycerol monoricinoleate

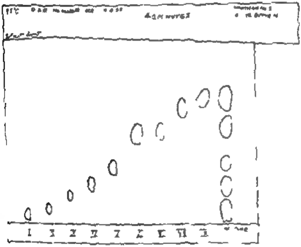
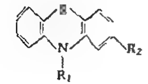
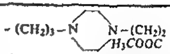
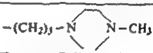
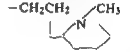


Fig 3 As for figure 1 except time = 40 minutes

In order to obtain these diagrams, the chromatograms were stained with iodoplatinate reagent The modified Marquis reagent was used for better differentiation and assistance in identification This is illustrated in figures 4 and 5

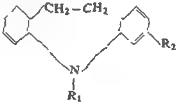
Table 1 lists the colours obtained with the various modified sulphuric acid reagents R_f values are not given, because it is better to run standards rather than to measure R_f values, especially when using both ascending and horizontal circular chromatography

Table I

Phenothiazine derivatives 	Trade names	R_1	R_2
Thiopropazate	Dartal		$-Cl$
Trifluperazine	Stelazine		$-CF_3$
Fluopromazine	Vesprin	$-(CH_2)_3-N(CH_3)_2$	$-CF_3$
Thioridazine	Mellaril		$-SCH$
Chlorpromazine	Largactil	$-(CH_2)_3-N(CH_3)_2$	$-Cl$
Mepazine	Pacatal		$-H$
Promethazine	Phenergan	$-CH_2-CH(CH_3)-N(CH_3)_2$	$-H$
Promazine	*Sparine*	$-(CH_2)_3\ N(CH_3)_2$	$-H$
Imipramine*	Tofranil	$-(CH_2)_3-N(CH_3)_2$	$-H$

ᵃ = a blue colour develops slowly after about 10 mins (if paper survives) ᵇ = colour fad rapidly, B = blue, BG = blue/green, Br = brown DB = dark blue, DG = dark greer DPu = dirty purple, fl = fluorescence, O = orange OBr = orange/brown, PG = pale greer Pk = pink, PO = pale orange, Pu = purple, PY = pale yellow, R = red, RO = redd s orange, Y = yellow, YG = yellow/green

* Not a phenothiazine, Imipramine is



Application to Biological Material

10 μl each of 1 per cent (w/v) solutions of fluopromazine, chlorpromazine and imipramine were added to and mixed with 10 ml of blood, which was then treated as described by DUBOST & PASCAL (1953) The washed, combined ether extracts were dried by shaking with solid sodium

254 mμ light	Modified conc H_2SO_4	Modified Marquis reagent	Modified Mandelin reagent	Modified Fröhde reagent	Modified Mecke reagent	50% HNO_3	Bromine water	1% $PdCl_2$
DB fl	R	R	O→R	R	R→Br	Nil	DG	RO
PY fl	PO	[illegible]	O	Pk	Pu	Nil	O[b]	[illegible]
PY fl	[illegible]	O	Br	O→R	O	Nil	O[b]	OBr
BG fl	B	BG →DPu	BG →RBr	B	Pu	Pu	PG	Br/Pu
YG fl	Pk	Pk	Br	R →Pu	Br	Pk[b]	G	Pk
B fl	O	RO	Br →RBr	Y →YG	Y →YG	PY	DG	Pu
B fl	R	RO	Br	Br	Br	BG[b]	Pu	Br
B fl	O	RO	BG	Y →YG	Y →YG	PY	DG	Br/Pu
R R	Nil	Nil[a]	Nil[a]	Nil	Nil[a]	DB →DG	Br	PO

sulphate and then evaporated to dryness on a boiling waterbath The cooled residue was dissolved in a few drops of chloroform and applied to the ester impregnated paper for chromatography The results are illustrated in Figure 6

The method of DUBOST & PASCAL (1953) was also applied in a slightly modified form to a 200 ml sample of urine (total volume 865 ml) collected over the 5 hours immediately after oral administration to a patient of 50 mg of triflupromazine (vesprin ®) The chromatogram showed two spots that gave a positive reaction with iodoplatinate reagent A control urine treated similarly gave no iodoplatinate positive spots

Figure 7 illustrates these results obtained from urine The spot with the higher R_f value was presumed to be the sulphoxide of fluopromazine, since the ultraviolet absorption curve of the extract displayed peaks corresponding to those of this metabolite

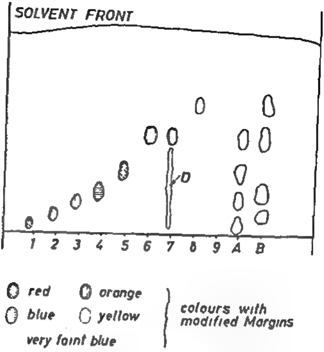


Fig 4 As for figure 3 but spots located by modified Marquis reagent
A = 1, 3, 5, 7, 9 B = 2, 4, 6, 8.
D = decomposition product

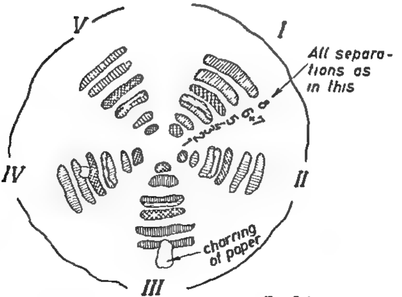


Fig 5A

Fig 5. Horizontal circular chromatography of the compounds listed in the legend to Figure 1 on Whatman No 3 paper impragnated with tributyrin (10% v/v solution in

Solve

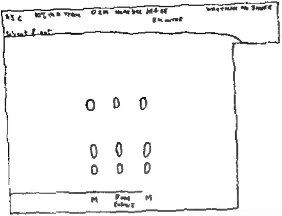
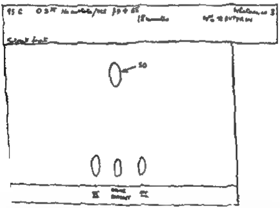


Fig 6 Ascending cylindrical chromatography of an extract of blood to which had been added a mixture of fluopromazine chlorpromazine and imipramine M = mixture of these three compounds Whatman No 3 paper impregnated with tributyrin (10% v/v solution in acetone) Time = 18 minutes
Solvent and temperature as for figure 1 Spots located with iodoplatinate reagent



g 7 Chromatography of extract of urine after ingestion of 50 mg of Triflupromazine esprin B) SO is probably the sulphoxide of Triflupromazine III = fluopromazine controls Conditions of chromatography as in figure 6

Summary

Rapid separation of 8 phenothiazine tranquillizers and imipramine ofranil R) occurs during reversed phase chromatography with aqueous olvents on ester impregnated paper at temperatures between 85 and 5 C The technique of this type of chromatography is simple and does ot require special apparatus The effects on R_f values of (a) type and mount of ester, (b) pH and concentration of solvent and (c) different

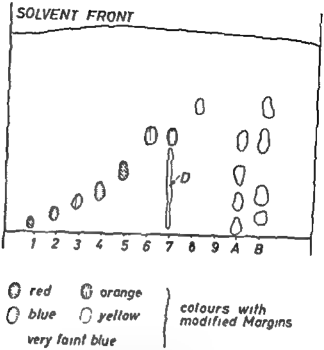


Fig 4 As for figure 3 but spots located by modified Marquis reagent
A = 1, 3, 5, 7, 9 B = 2, 4, 6, 8
D = decomposition product

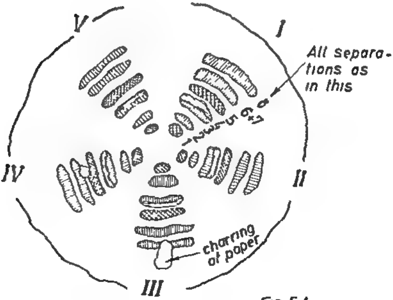


Fig 5A

Fig 5 Horizontal circular chromatography of the compounds listed in the legend to Figure 1 on Whatman No 3 paper impragnated with tributyrin (10% v/v solution in

Solve mins
4

Acta pharmacol et toxicol 1962, 19, 325–329

From the Department of Forensic Medicine, University of Edinburgh

The Rapid Separation of Drugs and Poisons by High Temperature Reversed Phase Paper Chromatography
3. Alkaloids

By

Harold V. Street

(Received October 15 1962)

Recent experiments have shown that mixtures of barbituric acid derivatives (STREET 1962a) and of phenothiazine tranquillizers (STREET 1962b) can be separated by high temperature reversed phase paper chromatography This technique has been used at 86° and 95° in a study of the behaviour of 31 alkaloids, whose R_f values (see table 1) have been investigated with solvents at six pH values ranging from pH 1 to pH 10 6

It is important to remember that, with this technique the R_f values serve only as a guide and are not to be relied upon as absolute measurements In any investigations of unknown compounds, control alkaloids must be run simultaneously alongside the mixture under investigation

Experimental details of the technique and a discussion of various factors influencing the movement of the applied compounds have been described previously (STREET 1962b) Tributyrin impregnated Whatman no 3 paper was used, and the spots were located first by inspection in 254 mμ light (Hanovia 'Chromatolite') and then by dipping in iodoplatinate reagent Sometimes the chromatograms were cut so as to divide the separated spots into two parts This procedure enabled the classical alkaloid reagents (as modified by STREET, 1962b) to be used directly on the paper, thus providing additional identification

This technique requires only 17 minutes for the chromatographic run and can give a useful range of R_f values with various pH values of the solvent

Three analgesic drugs bearing a close chemical structural relationship, i e dextromoramide, dipipanone and methadone (see fig 1) are separated

temperatures have been studied. A modified Marquis reagent, and other locating reagents containing concentrated sulphuric acid, are described for direct use on the ester-impregnated paper. Examples are also included of the application of the technique to blood and urine extracts. The time required for a chromatographic run is short, from 20 minutes for ascending and to 40 minutes for circular chromatography.

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clearly in 17 minutes with a solvent of pH 4 58 at 95° A tracing of the chromatogram stained with iodoplatinate is shown in fig 2

A preliminary screening test has been described in a previous communication (STREET 1962c) If this screening test reveals a compound in the alkaloid fraction that does not move from the origin, the chromatography should be repeated with buffers of pH 4 58 and pH 10 6 Reference to table 1 will then provide information that, together with ultraviolet absorption curves, may be sufficient to identify the compound

Table 1

R_f values of 31 alkaloids subjected to chromatography at 86° and at 95° on tributyrin-impregnated Whatman No 3 paper with solvents at various pH values Alkaloid spots located by 254 mμ light, by iodoplatinate reagent and by a modified Marquis reagent

Time of run = 17 minutes

S = compound streaks over the chromatogram Y = yellow B = blue, Pu = purple, Br = brown Or = orange

* = Not detected at these pH values

		pH ca 3	pH 3 3	pH 4 58	pH 7 4	pH 10 3	pH 10 6	Colour with Mod Marquis
Amethocaine	86°	91	77	77	3	[illegible]	–	–
	95°	98		63	5	–	0	–
Atropine	86°	91	90	90	84	–	–	–
	95°	97	–	90	75	–	25	–
Berberine	86°	30	28	26	23	18	–	Y
	95°	37		32	30	–	22	–
Brucine	86°	82	76	76	45	25	–	Y
	95°	83	–	76	45	–	28	–
Butaca ne	86°	93	81	75	1	3	–	–
	95°	95	–	63	5	–	0	–
Cocaine	86°	89	86	86	9	[illegible]	–	–
	95°	95	–	[illegible]	8	–	0	–
Code ne	86°	94	88	95	66	35	–	B/pu
	95°	97	–	[illegible]	55	–	39	–
Cotarnine	86°	[illegible]	86	86	86	75	–	Y
	95°	95	–	87	87	–	78	–
Cycl zine	86°	91	88	75	S	0	–	–
	95°	95	–	58	3	–	[illegible]	–
Dextromoramide	86°	–	–	–	–	–	–	–
	95°	85	73	23	0	–	–	–
D p panone	86°	82	74	47	1	3	–	–
	93°	86	–	33	2	–	0	–
Emetine	86°	–	–	–	–	–	–	–
	95°	98	–	86	6	–	0	–

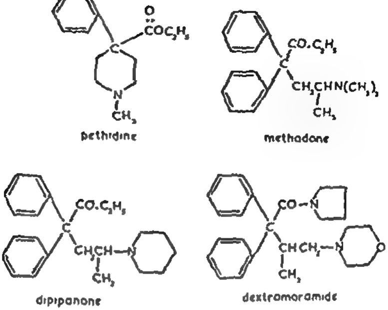
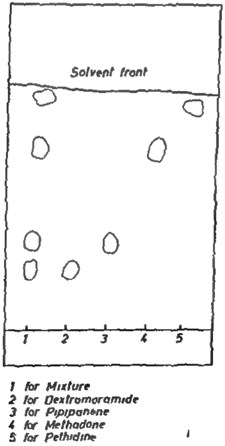


Fig 1 Structural formulae of pethidine, methadone, dipipanone and dextromoramide



Preparation of Standards and of Buffer Solvents

The alkaloid standards were prepared as 2% (w/v) solutions in ethanol Compounds not readily soluble in ethanol were dissolved in chloroform

pH 1 – *0 1 N sulphuric acid solution was used to give a pH of between* 1 and 1 5 units Papers that have been run in this solvent must not be heated during drying because the residual concentrated acid will char the paper

pH 3 3 and pH 4 58 – M/5 sodium acetate – hydrochloric acid

pH 7 4 – M/15 phosphate

pH 10 3 – M/10 glycine – NaCl – NaOH

pH 10 6 – M/20 carbonate – borate

Summary

The behaviour of 31 alkaloids during high temperature reversed phase chromatography has been investigated Rf values at 86° and 95° with solvents of 6 pH values ranging from pH 1 to pH 10 6 are given Chemical compounds closely related may be clearly separated by this method

Acknowledgement

The research programme on which this paper is based was supported by a grant from the Scottish Hospital Endowments Research Trust

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Table 1,

		pH ca 1	pH 3 3	pH 4 58	pH 7 4	pH 10 3	pH 10 6	Colour with Mod Marquis
Ethyl Morphine	86°	90	89	89	42	22	–	Pu
	95°	94	–	89	34	–	20	–
Homatropine	86°	–	91	90	86	•	•	–
	95°	98	–	–	–	–	–	–
Hyoscine	86°	93	90	94	59	•	•	–
	95°	–	–	–	–	–	–	–
Lidocaine (lignocaine)	86°	90	89	80	8	4	–	–
	95°	95	–	74	5	–	4	–
Methadone	86°	93	76	75	1	0	–	–
	95°	88	–	67	2	–	0	–
Morphine	86°	90	87	94	90	67	–	Pu
	95°	94	–	89	76	–	87	–
Nalorphine	86°	90	85	93	46	42	–	Pu
	95°	94	–	85	41	–	71	–
Narceine	86°	97	94	97	–	88	–	Y
	95°	93	–	90	89	–	96	–
Narcotine	86°	90	69	21	0	0	–	–
	95°	93	–	13	0		0	–
Nicotine	86°	91	90	90	54	36	–	–
	95°	98	–	91	47	–	–	–
Nupercaine	86°	87	68	50	0	0	–	–
	95°	90	–	44	1	–	0	–
Papaverine	86°	85	71	22	2	2		Br
	95°	89	–	14	2	–	3	–
Pethidine	86°	87	87	87	14	2	–	*Or*
	95°	96	–	86	9	–	–	–
Physostigmine	86°	90	88	92	33	S		Y
	95°	94	–	90	27	–	9	–
Pilocarpine	86°	93	95	95	77	72	–	–
	95°	–	–	89	76	–	–	–
Quinine	86°	92	83	74	13	6	–	–
	95°	94	–	69	11	–	[illegible]	–
Sparteine	86°	–	92	96	92	S	–	–
	95°	96	–	87	80	–	–	–
Strychnine	86°	83	76	76	37	18	–	–
	95°	85	–	79	33	–	21	–
Yohimbine	86°	75	95	97	8	6	–	–
	95°	82	–	67	11	–	6	–

Table 1

Rf values of nornicotine and its derivatives Ascending run over 12 hrs at 23° on SS 2045 b paper treated with 2 M phosphate buffer Solvent A butanol acetic acid water (4 1 5) Solvent B tert amyl-alcohol water ethyl – p-aminobenzoate (100 20 0 3) Staining with CNBr + p aminobenzoic acid

Drug	Colour	Rf values	
		Solvent A	Solvent B
Nornicotine	Orange	0 39	0 13
Nicotine	Yellow	0 46	0 55
Ethylnornicotine	Yellow	0 54	0 61
Allylnornicotine	Yellow orange	0 69	0 90
Acetylnornicotine	Red	0 75	0 93
Carbaminoyl nornicotine	Red	0 65	0 93
Benzoylnornicotine	Red	0 91	0 95

This tallies with the results of POLONOVSKI & POLONOVSKI (1927) who reported their

Carbaminoylnornicotine was a slightly yellowish substance m p 147–149, yield 35 per cent It was precipitated 4 times from a chloroform solution with petroleum spirit

Benzoylnornicotine was synthesized by HUCKER s method (1958 and 1961) with the nicotine nornicotine mixture (10 1) as the starting material This alkaloid mixture was obtained from Kentucky Burley tobacco by percolating it with acidulated water, extracting the concentrated percolate with ether and distilling the extract The benzoylnornicotine was a viscous yellow liquid b p 225 226°/1 mm Hg after several distillations

All these nornicotine derivatives were studied by paper chromatography The sensitive colour reaction of isatin (STEPHENS & WEYBREW 1959) showed minimal amounts of nornicotine as an impurity in both the ethyl and carbaminoyl derivatives No other pyridine impurities were found The Rf values are given in table 1 In a solvent of butanol acetic acid water the Rf values increase from that of nornicotine to that of allylnornicotine as the N alkyl chain lengthens

The LD50 doses for nicotine tartrate nornicotine and the synthesized nornicotine derivatives were determined The compounds were dissolved in water and the solutions were neutralized and injected intraperitoneally into groups of 10–12 white mice weighing 15–20 g The LD50-values were determined by the graphical method of MILLER & TAINTER (1944)

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From the Department of Pharmacology, University of Helsinki

The Lethal Dose, Pressor Effect and Intestinal Activity of some *Pyrrolidine N-substituted Nornicotine Derivatives*

By

M. Mattila and A. Vartiainen

(*Received August 20, 1962*)

The pharmacodynamic actions of nicotine are more potent than those of its derivatives and related alkaloids (LARSON *et al* 1961, WERLE & SCHIEVELBEIN 1961) The effects are diminished if the pyrrolidine ring of the nicotine molecule is N-demethylated or opened and are further decreased as the side chain produced by the opening of the pyrrolidine ring is shortened If this side chain is de-aminated, the convulsive effect is replaced by sedation (LARSON *et al* 1946) The effects of nornicotine, though qualitatively similar, are quantitatively weaker than those of nicotine (HICKS *et al* 1935 and 1947, KITAMURA 1958, YAMAMOTO *et al* 1957 and 1958) It is suggested that nicotine and nornicotine act on the same or similar receptors (YAMAMOTO *et al* 1958)

The purpose of the work reported here was to investigate changes in the effects of nornicotine after the hydrogen atom attached to the pyrrolidine N-atom had been replaced by various radicals While this papers was being written, it has been reported (REES & SCHNIEDEN 1962) that allylnornicotine is less toxic than nicotine and nornicotine and lacks the antidiuretic effect characteristic of nicotine

Material and Methods

Except for benzoylnornicotine, the nornicotine derivatives were synthesized from 1 nornicotine (Fluka AG) by v BRAUN & WEISSBACH s methods (1930)

Ethylnornicotine was a colourless liquid, b p 100–101 °/2 mm Hg yield 10 per cent m p of picrate, 165°

Allylnornicotine was a colourless liquid b p 128–130/9 10 mm Hg yield 10 per cent, m p of picrate 179–181° The allyl test with phloroglucine was weakly positive

Acetylnornicotine was a colourless viscous liquid, b p 182–185/2 mm Hg yield 70 per cent Its picrate separated from alcohol as a solid but soon turned into an oil

Table 3

The relative potencies of nornicotine and some derivatives on the isolated guinea pig ileum Each drug had been compared with nicotine with µmol as the weight unit

Drug	Relative doses causing an equal response		
	Without antagonist	Thiamine 200 µg/ml	Hexamethonium 50 µg/ml
Nicotine	1	1	1
Nornicotine	13 10 12.5 13	15 23 12.5	17 14.8 17.5
Ethylnornicotine	8.5 5 6.5 3.8	5 4 5.5	10 [illegible] 5.5
Allylnornicotine	50 33 41 52	no response	no response
Acetylnornicotine	150 167 117	110 165 125	130 120
Benzoylnornicotine	72 86 70 87	no response	no response
Carbaminoyl nornicotine	300 270 350	no response	no response

relative potencies of nornicotine and its derivatives in various conditions, compared with nicotine The effects of allylnornicotine and benzoyl nornicotine in particular were modified by antagonists They caused no more contractions and even enhanced the effect of the antagonists by preventing nicotine induced contractions Such inhibitory action against nicotine appeared only in the presence of a nicotine antagonist

All the nornicotine derivatives except acetylnornicotine, caused the atropinized (3 µg/ml) guinea pig ileum to contract, though showing no response to 100-fold doses of acetylcholine This dose of atropine inhibited contractions caused by histamine and 5HT more effectively (3 fold) that it did nicotine contractions (1.5 fold) Atropine and phentolamine together (3 µg/ml each) caused a marked inhibition to nicotine, but a much smaller one to histamine and 5HT

All the drugs studied stimulated the isolated ileum of the reserpinized (2 mg/kg) guinea pig These contractions were effectively inhibited by atropine (100-fold inhibition) The inhibitory action of atropine was much less effective against contractions caused by 5HT and histamine

C *Blood Pressure of the Cat*

Except for acetyl nornicotine all the drugs caused a rise of blood pressure in the anaesthesized cat Within certain limits the response was proportional to the dose When the drugs were injected at 10 *min intervals* the response showed no tachyphylaxis Pressor response was encountered with contraction of the nictitating membrane Such contraction was also caused by acetylnornicotine, which decreased the blood pressure Contrary to its weak intestinal action benzoylnornicotine showed a strong

Table 2

The LD50 for nornicotine and its derivatives after i p injections
Mean survival times after administration of doses near the LD50

Drug	LD50		Mean survival time min
	mg/kg	mmol/kg	
Nicotine	10	0 061	1 5
Nornicotine	33	0 221	5 5
Ethylnornicotine	38 5	0 218	4 6
Allylnornicotine	60	0 318	3 8
Acetylnornicotine	395	2 09	17 1
Benzoylnornicotine	300	1 178	22 7
Carbaminoyl nornicotine	>1000	>5 22	-

Results

A *Acute Toxicity*

The LD50 doses for nornicotine and its derivatives after i p injection are shown in table 2, which also gives the mean survival times after injecting doses near to the LD50 The LD50 for nicotine has been cal culated for the base

The effects of nicotine, nornicotine, ethylnornicotine and allylnornico tine were qualitatively similar Their administration resulted in excitatory symptoms, and they increased spontaneous motility and provoked con vulsions The convulsions caused by *acetylnornicotine* were relatively mild It also decreased spontaneous motility *Benzoylnornicotine* showed a clear sedative effect After doses of about the LD50 the mice became somnolent, and the straightening reflex disappeared 5–10 min before death The convulsions were mild and tonic in type The toxicity of *carbaminoylnornicotine* was minimal Doses of 1000 mg/kg caused no visible effect except a slight decrease in spontaneous motility

A small dose of nicotine proved to be the best inhibitor against a larger dose of nicotine injected later No other nornicotine derivative counter acted the lethal action of 20 mg/kg of nicotine (twice the LD50)

B *The Guinea Pig Ileum*

Nornicotine and its derivatives caused the isolated guinea pig ileum to contract, the strength of the contractions being proportional to the dose Cocaine (5 μg/ml), hexamethonium (50 μg/ml) and thiamine (200 μg/ml) inhibited contractions so effectively that 10 fold doses of nicotine were needed to effect the original response Acetylcholine contractions were also inhibited by thiamine, but not to the same degree Table 3 shows the

pathetic ganglion Possibly it had also a muscarinic action, because it provoked a slight depressor effect on the blood pressure of the cat after pre treatment with tetraethylammonium, which abolished any nictitating response even to electrical stimulation of the cervical sympathetic nerve The strong inhibitory action of atropine on contractions of the guinea-pig ileum caused by acetylnornicotine is another indication of the parasympathetic nature of its action

Further investigations with nornicotine derivatives, including enzyme studies, are proceeding

Summary

Ethyl , allyl , acetyl , benzoyl and carbaminoylderivatives of nornicotine were synthesized and tested pharmacologically

1) LD50 (mg/kg) was 10 for nicotine, 33 for nornicotine, 38 5 for ethylnornicotine, 60 for allylnornicotine, 300 for benzoylnornicotine, 395 for acetylnornicotine and >1000 for carbaminoylnornicotine Convulsions, if present, were mild for acetyl and benzoyl derivatives The only effect of carbaminoylnornicotine was a slight sedation

2) All the nornicotine derivatives contracted the isolated guinea pig ileum These contractions were inhibited by cocaine, hexamethonium, or thiamine but hardly at all by atropine, except those caused by acetylnornicotine Phentolamine or pre treatment of the animal with reserpine greatly enhanced the effect of atropine

3) Acetylnornicotine had a depressor effect on the blood pressure of cat, the other drugs had a pressor effect All the drugs caused a simultaneous contraction of the nictitating membrane The effects were almost totally abolished by hexamethonium or tetraethylammonium After this treatment acetylnornicotine still caused a slight depressor effect, but this was abolished by atropine After both these treatments nicotine and ethylnornicotine still caused a slight rise in blood pressure

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pressor activity (1/10 of nicotine effect) The pressor effect of allylnornicotine was rather weak (1/200 of nicotine effect)

After pretreatment with hexamethonium or tetraethyl ammonium all the effects on blood pressure were almost completely eliminated Acetylnornicotine still caused a slight decrease, which was abolished by atropine Only nicotine and ethylnornicotine caused any rise in pressure after pretreatment with hexamethonium and atropine

Discussion

In our experiments, nicotine was the most potent nornicotine derivative Both acute toxicity and the pressor and intestinal activities were reduced if the pyrrolidine ring of the nicotine molecule was N-substituted with another radical or N-demethylated Nicotine was found to give quaternary ions at physiological pH (WICKERY & PUCHER 1929) For this reason, TAYLOR (1951) and BARLOW & HAMILTON (1962) consider the effects of nicotine *in vivo* due, in part at least, to the pyrrolidinium-ring of its molecule If so, the methyl group is obviously the most effective radical for the ionization of the pyrrolidyl N-atom

The formation by nornicotine of amides with organic acids decreased or abolished the convulsive symptoms, possibly owing to decrease of the ionization of the pyrrolidine ring by the acid radical, which may lower the alkalinity of the amine, thus producing a kind of "deamination" This tallies with the results of LARSON *et al* (1946), which show that pyridine compounds produce convulsive effects only if they have an amino group in the side chain, otherwise they have a sedative effect The effects of nornicotine were particularly reduced when it was converted to it's carbaminoyl derivative It should be noted here that the $-CO-NH_2$ group is an essential structural component of many sedatives and hypnotics

We found no antagonism between nicotine and some other nornicotine derivatives similar to what exists in the morphine group It is probable that not only the *activity* of nicotine but also its *affinity* for the receptor are greater than those of its analogues The weak antagonism in the guinea-pig ileum is difficult to explain It can hardly be due to an unspecific toxic effect because the same phenomenon did not appear when equal doses were administered without hexamethonium and thiamine

The poor inhibitory effect of atropine on nicotine action in the guinea-pig ileum was surprising, as were the effects of sympatholytic agent and reserpine Since they greatly enhanced the inhibitory effect of atropine, it seems obvious that the nicotine-induced contractions of the guinea-pig ileum are partly transmitted via sympathetic nerve endings

Acetylnornicotine seemed to have a marked effect on the parasym-

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From the Biological Department, the Pharmaceutical Institute
University of Oslo

Pharmacological and Chromatographic Differentiation between a Secondary Kinin from Human Urine and Bradykinin

By

Kjell Briseid Jensen and Anne Marie Venneröd

(Received July 13 1962)

A method has previously been described for preparing a concentrate of the main kinin of human urine (BRISEID JENSEN & VENNEROD 1962 a) The active principle could be distinguished from bradykinin neither in paper chromatographic experiments nor in parallel pharmacological assays (BRISEID JENSEN & VENNEROD 1962 b) Another active fraction was to a large extent removed from the main urinary kinin during purification The presence of small quantities of that fraction might give origin to the small secondary peak of activity detected in the paper chromatograms of the main urinary kinin It might also be the cause of the small difference in index of discrimination observed when bradykinin and the main urinary kinin were tested simultaneously on the isolated rat uterus and on the rat blood pressure preparation (BRISEID JENSEN & VENNEROD 1962 b)

Some previous chromatographic investigations also indicated that more than one active substance must be present in urine extracts (WALASZEK 1957, BRISEID JENSEN 1958) though other chromatographic examinations did not show any heterogeneity (GOMES 1955, GADDUM & HORTON 1959) In counter-current distribution experiments GOMES (1957) separated bradykinin and the active principle of urine extracts

Previous pharmacological parallel assays have given no clear evidence of a dissimilarity between bradykinin and the urine extracts tested (GOMES 1955, GADDUM & HORTON 1959, HORTON 1959), only GOMES (1957) found minor differences

We describe here a method for preparing a concentrate of a secondary kinin from human urine As was done with the main urinary kinin (BRISEID JENSEN & VENNERÖD 1962 b), the substance was compared with bradykinin both by paper chromatography and in pharmacological parallel tests For practical reasons the bradykinin like main urinary kinin has in this paper been designated Z_1 and the secondary kinin Z_2

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B Chromatography

Descending chromatography with Whatman No 1 filter paper was used The solvent was N butanol, acetic acid, water (40 + 10 + 50 vol), and the procedure was as described previously (BRISEID JENSEN & VENNERØD 1962 b)

2. Comments on the Technique

A Preparation of a Concentrate of Z_2

1 *Distribution of Z_1 and Z_2 by the butanol extraction procedure* It has been shown previously (BRISEID JENSEN & VENNERØD 1962 b) that nearly half of the activity left in the sodium chloride saturated aqueous phase after butanol extraction at pH 1 5 could be shaken into butanol if hydrochloric acid was again added to give a pH of 1 5 In the same experiment it was shown that bradykinin was almost completely transferred to butanol by the extraction procedure suggested If it is assumed that Z_1 is identical with bradykinin (BRISEID JENSEN & VENNERØD 1962 b), or that it has almost the same partition coefficient in the liquid system used, it means that no Z_1 will be present in the water phase used for preparing the Z_2 It also means, however, that roughly half of the Z_2 originally present will be lost by the butanol extraction To test whether bradykinin, and accordingly probably also Z_1, could be quantitatively transferred to butanol at a higher pH with more Z_2 remaining in the water phase, model experiments were carried out with bradykinin and Z_2 dissolved in water at

Table 1

Recoveries by butanol extraction from water of bradykinin and of Z_2 at different pH values

Test solutions
A [illegible]
[illegible] μg
D 100 ml sodium chloride saturated hydrochloric acid of pH 2 4 + 20 μg bradykinin.
E 100 ml sodium chloride saturated hydrochloric acid of pH 2.7 + 100 μg bradykinin.
Activities are given as μg bradykinin and obtained in rat uterus assays for further details see text

Test solution	pH	Activities				
		Start	Transferred to butanol		Left in water phase	
		μg	μg	%	μg	%
A	1 5	40	12 7	32	14 4	36
B	1 5	100	70 6	71	<0 8	<1
C	2 4	20	4 2	21	13 6	68
D	2.4	20	17 8	89	0 7	4
E	2 7	100	74 1	74	12 0	12

1. Technique

A Materials and Assay.

I *Standard preparation* In the paper chromatographic experiments both the bradykinin fractions and the fractions from the secondary kinin (Z_2) runs were assayed against synthetic bradykinin[1]) In the pharmacological experiments no standard preparation was needed, the comparison between the effects of bradykinin and of Z_2 on different biological preparations being emphasized The figures given for relative activities of bradykinin and of Z_2 in the pharmacological experiments were based on rat uterus assays carried out simultaneously

II *Test preparations* The same batch of bradykinin used as standard substance was also used as test substance in the experiments The concentrates of Z_2 were prepared as described below

III *Urines* For the preparation of Z_2 concentrates the pooled 24 hour urines from 4–5 individuals (males and females) and day urines from several males were used The urines were frozen and stored at −17° until used

IV *Assays* The fractions from the paper chromatograms were estimated by comparing the unknown with two standard doses, the standard dose-ratio usually being 3 2 For the pharmacological parallel assays (2 + 2) or ' bracketing" assays were used

B Preparation of a Concentrate of Z_2

The first stages of the purification procedure were as described previously for the main urinary kinin, Z_1, (BRISEID JENSEN & VENNEROD 1962 a)

When the amount of Z_1 present in the eluate from Amberlite CG 50 had been transferred from water saturated with sodium chloride to butanol by shaking at pH 1 5, significant amounts of Z_2 were left in the water phase The further purification of Z_2 was as described below, the figures given referring to a 50-litre batch of urine

The active solution (500 ml) was concentrated to a small volume at about 30° under reduced pressure (5–10 mm Hg) on a rotating evaporator To the highly viscous fluid were added 400 ml of concentrated acetic acid, and the mixture was filtered through filter paper When the sodium chloride in the flask and on the filter paper had been washed with 5 × 20 ml of acetic acid, the filtrates were evaporated to dryness at about 30° and 5–10 mm Hg The last traces of water and acetic acid were removed at room temperature at a low pressure (0 005–0 01 mm Hg) For further salt removal the active substance was eluted with 20 ml and then with 4 × 10 ml of concentrated acetic acid The combined acetic acid fractions were filtered through filter paper, and the acid was removed on a rotating evaporator The remaining water and acetic acid were removed at room temperature at 0 005–0 01 mm Hg The concentrate was kept over silica gel in an evacuated desiccator at room temperature

C Pharmacological Experiments

For the pharmacological parallel assays the biological effects used were stimulation of the isolated rat uterus, stimulation of the isolated guinea pig ileum, relaxation of the isolated rat duodenum, rat blood depressor effect The techniques were as previously described (BRISEID JENSEN & VENNERÖD 1962 b)

1) Bradykinin ampoules containing 100 µg/ml were kindly furnished by Sandoz A G, Basel, Switzerland

Table 3

Paper chromatography of bradykinin and Z_2

Liquid system N butanol acetic acid water (40 + 10 + 50 vol)
[illegible]
[illegible]perature 22–24
Chromatogram run for 13½ hours
Length of run 30 cm

Fraction number	Recovery %		
	Bradykinin	Z_2	Bradykinin + Z_2
1	–	–	–
2	–	0 3	–
3	–	1 5	1 0
4	–	4 4	2 9
5	–	6 6	6 8
6	–	5 8	2 0
7	–	3 2	2 9
8	–		–
9	–	–	–
10	0 6	0 5	0 8
11	1 6	0 3	3 7
12	2 6	0 3	3 7
13	9 6	–	1 8
14	5 8	–	–
15	–		–
16	–	Not tested	–
17	–	Not tested	–
18	–	Not test d	–
19	–	Not tested	–
20–30	Not tested	Not tested	–
Sum	20 2	22 9	25 6

B *Chromatography*

Table 3 gives the results from a paper chromatographic run of Z_2, bradykinin and a mixture of the two substances The results are calculated as percentage recoveries

4. Comments on the Results

A *Pharmacological Assays*

The comparative assays on different biological preparations showed that Z_2 is not identical with bradykinin The combined use of rat uterus and rat duodenum, which was suggested by HORTON (1959) for the differentiation of kinins and other oxytocic peptides, did not distinguish between bradykinin and Z_2 The latter substance, however, seemed to be considerably more active than bradykinin when assayed on the guinea pig ileum or by its rat blood depressor effect than on the rat uterus The

different pH-values The results are shown in table 1 The figures for pH 1 5 are the same as those previously given (BRISEID JENSEN & VENNEROD 1962 a), and the extraction procedure was as described in that paper The Z_2 preparations used were made by the procedure mentioned in this paper Table 1 shows that an increase in pH from 1 5 to 2 7 will decrease the purity of the Z_2 concentrates, as a significant amount of bradykinin (12%) remained in the water phase In an experiment (not included in the table) at pH 3 9 about one-third of the bradykinin remained in the water solution Although pH 2 4 seemed more favourable, the Z_2 concentrates used for the present work were prepared by butanol Z_2 extraction at pH 1 5 to secure the highest possible degree of purity It should also be remembered that urine usually contains considerably more Z_1 than Z_2, which will proportionally decrease the purity of the Z_2 concentrates from extractions at pH-values over 1 5

II *Salt removal and loss of activity* The suggested acetic acid salt removal procedure gave Z_2 concentrates with roughly the same content of sodium chloride as the Z_1 purification method (BRISEID JENSEN & VENNEROD 1962 a), namely 30–60 mg corresponding to a 50 litre batch of urine The losses of activity occurring in the residual water phase after butanol extraction up to the final acetic acid solution ranged from 10 to 20%

3. Results

A *Pharmacological Assays*

Results of comparative assays with Z_2 and bradykinin on different biological preparations are given in table 2 The figures recorded were partly obtained in (2 + 2) assays with 6 series of 4 randomized doses, partly by "bracketing" the unknown between two standard doses The statistical procedure described by SCHILD (1942) was used for the treatment of the results of the (2 + 2) assays

Table 2

Pharmacological parallel assays

Ng bradykinin equi active with the amount of Z_2 corresponding to 1 ng bradykinin when tested simultaneously on rat uterus

Biological effect	ng bradykinin (Index of discrimination)	Fiducial limits %, P = 0 05 Rat uterus	—
Guinea pig ileum stimulation	11 9	92–107	96–104
Rat duodenum relaxation	1 2	96–104	*
Rat blood depressor effect	15	*	*

* Result based on "bracketings"

As mentioned in the introduction to this paper, previous chromatographic work (WALASZEK 1957, BRISEID JENSEN 1958) also produced some evidence of more than one active substance being present in urine extracts, though other chromatographic investigations did not detect any heterogeneity (GOMES 1955, GADDUM & HORTON 1959) WALASZEK (1957) not only observed more than one peak of activity in column chromatographic runs of urine extracts but could also detect parallel peaks with bradykinin His paper chromatograms, however, showed no heterogeneity of the two substances In counter current distribution experiments GOMES (1957) noticed a separation of bradykinin and the active principle of the urine extract used

The discrepancies in the results mentioned above may be partly due to the varying relative amounts of Z_1 and Z_2 present in urines and to differences in the purification procedures used A low degree of purity of the earlier bradykinin preparations would also add to the separation difficulties

The main previous pharmacological examinations have given no evidence of a dissimilarity between bradykinin and the urine concentrates used Thus GOMES (1955), GADDUM & HORTON (1959) and HORTON (1959) could not distinguish between the two substances in parallel assays, in a later paper, however, GOMES (1957) reports minor differences It should be mentioned that HORTON (1959) only used the combination of rat uterus and rat duodenum for differentiation between kinins and other active substances and pointed out that it did not distinguish between bradykinin, wasp kinin and urinary kinin In the work reported here rat uterus and rat duodenum could not differentiate between bradykinin and Z_2 but Z_2 proved to be considerably more active than bradykinin when tested either on the guinea pig ileum or by rat blood pressure than on the rat uterus

Summary

A method has been described for preparing a concentrate of a secondary kinin from human urine (Z_2) The procedure has much in common with a method previously developed for the preparation of a concentrate of the main kinin from human urine (Z_1) (BRISEID JENSEN & VENNERØD 1962 a)

A comparison was made between synthetic bradykinin (Sandoz A G, Basel Switzerland) and a concentrate of Z_2 Pharmacological parallel assays with the rat uterus on the one hand and guinea pig ileum or rat blood pressure on the other, both distinguished between the substances Indices of discrimination of 12 and 15 respectively were obtained Parallel assays with rat uterus and rat duodenum failed to discriminate between

results on blood pressure were not surprising, even a preparation of Z_1 contaminated with about 30% of Z_2 gave an index of discrimination significantly differing from 1 in the same tests (BRISEID JENSEN & VENNERÖD 1962 b) The result recorded in table 2 for a guinea pig ileum and rat uterus assay, however, was more remarkable, as no difference had previously been detected with the same Z_1 preparation as that mentioned above and used in the blood pressure assay The explanation could be that the purification procedure for Z_2 is not so effective as that for the Z_1 preparations and that other stimulants of guinea pig ileum present could accordingly add to the ileum effect The fact that another batch of Z_2, prepared from other urines and by a different method also gave an index of discrimination of the same order makes the objection less important It should be mentioned that the contracting effect of Z_2 on guinea pig ileum seemed to be somewhat more rapid than that of bradykinin

In the rat blood depressor tests it was difficult to obtain responses stable enough for (2 + 2) assays To save Z_2 material the rat preparations were stabilized through several bradykinin doses When Z_2 was then given, the effect seemed to be much more marked at first, then wearing off to a more stable lower level Initial indices of discrimination roughly estimated to be about 30 were thus obtained

B *Chromatography*

The acetic acid butanol system was chosen for the paper chromatography, as it had previously (BRISEID JENSEN & VENNERÖD 1962 b) separated a Z_1 preparation into two active fractions The main fraction passed together with bradykinin, but a small fraction had a shorter run The results given in table 3 show that bradykinin and Z_2 were completely separated and that the short running substance previously observed must be identical with Z_2

5. Discussion

The pharmacological parallel assays, as well as the chromatographic investigation, showed that substance Z_2 is not identical with bradykinin As it has previously been shown that Z_1 could not be distinguished from bradykinin in the same tests, the difference between Z_1 and Z_2 is also clearly established The degree of purity of the Z_2 preparations may be open to question The paper chromatographic results, however, prove that it is practically free from Z_1, and its pharmacological properties suggest that it must be considered a new urinary kinin It might, as also does Z_1, contain other kinins which are too similar to be detected by the chromatographic method employed

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B. *Chromatography.*

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From the Biological Department the Pharmaceutical Institute
University of Oslo

The Smooth Muscle Relaxing Effect of Thyme (Thymus vulgaris L)

By

Kjell Briseid Jensen and Olav Kjell Dyrud

(Received July 13 1962)

In a short communication LENDLE & LU FU HUA (1937) reported that extracts of fresh thyme inhibited pilocarpine induced spasms in the lungs of spinal cats Extracts of the dried herb had no such effect The active principle could be transferred from water to diethyl ether at alkaline pH and from the organic phase to acidified water

Apparently no other studies on the spasmolytic properties of fresh thyme are available We have therefore investigated the effect of thyme, in which the enzymes had been inactivated during the drying process, on various types of isolated smooth muscles and against different stimulants

1 Technique

A *Thyme Extracts*

The extracts used for our work were prepared from thyme that had been quickly dried by infra red irradiation Alcohol stabilised herb and herb dried at 20–25° were also used in preliminary experiments[1]) The active principles were purified by the method described by SCHILL & ÅGREN (1951) for the assay of Belladonna alkaloids In order to remove completely the volatile oil, the extracts were finally dried at about 0 01 mm Hg

B *Biological Preparations*

1 *Guinea pig ileum* A guinea pig (usually weighing 200–300 g) was killed by a blow on the head and bled A 3–4 cm segment of terminal ileum was suspended in Tyrode solution maintained at 32 33° (0 1) If spontaneous contractions were troublesome, the concentration of Ca + in the solution was decreased by a half A Schild lever was used the load ranged from 0 7 1 1 g The period of contact of active solution

1) The authors are much indebted to Axel Hopstock Chemist of Kragerø for supplying thyme that had been dried in various ways

the two kinins In paper chromatographic runs bradykinin and Z_2 were completely separated As it has previously been shown (BRISEID JENSEN & VENNERÖD 1962 b) that the main urinary kinin (Z_1) could not be distinguished from bradykinin in the pharmacological and chromatographic tests described in this present paper, the difference between Z_1 and Z_2 was clearly established

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2. Comments on the Technique

Thyme extracts The fresh thyme lost about 75% of its weight during drying, the doses of thyme mentioned in this paper refer to the dried herb The drug concentrates were kept over silica gel in vacuum desiccators The active principle(s) seemed to be stable under such conditions After a year about the same concentrations as originally were needed to inhibit the acetylcholine induced contractions of guinea pig ileum by about 50%. When Tyrode solutions of thyme concentrates were kept at 4°, the activity remained the same for at least 3 weeks

As mentioned by LENDLE & LU FU-HUA (1937), the activity of thyme herb dried without special precautions was low In our experiments we had to use about 10 times higher concentrations of herb dried at 20–23° than of herb of the same batch dried by infra red irradiation The thyme concentrates obtained by extracting with boiling ethanol during addition of calcium carbonate were highly active

3. Experimental

Comparison of the inhibitory actions of thyme on different agonists To obtain a rough estimate of the effectiveness of inhibition we attempted to establish the thyme concentration needed to reduce a submaximal contraction of an agonist by about 50% (40–60%) This rather primitive way of judging the potency of an antagonist was nevertheless considered satisfactory for the present experiments on relatively impure drug extracts In order to eliminate to some extent the effect of the differences between muscles the agonists were compared in pairs (sometimes in threes) on the same biological preparation Acetylcholine was used in all the experiments as a "standard" agonist In the experiments on guinea pig ileum, rat uterus and rat duodenum the addition of antagonist was always repeated for the number of cycles necessary to develop maximum effect The usual design of an experiment on three types of biological preparations was as described below As soon as the muscle had become stable on submaximal doses of the agonists in question lower concentrations of the agonists were tested to judge the slope of the dose effect curves Large differences in slope would make the comparisons of inhibition correspondingly inaccurate Thyme was then added as antagonist at a concentration that decreased by about 50% the submaximal contraction of the agonist most easily inhibited (acetylcholine) Then the thyme concentrations were increased so as to reduce, if possible, the contractions of the other agonist also

Because of technical difficulties caused by a considerably longer cycle

varied from 15–40 seconds, and the interval between doses was fixed within the range of 3 to 4 minutes The thyme was added as antagonist 1½ to 2 minutes before adding the agonist

In some of the experiments we used a 10 ml organ bath, in which Tyrode solution could replace the fluid by means of upward displacement and overflow Contractions were produced by manual addition of volumes ranging from 0 2 to 0 5 ml from 1 ml graduated syringes Washing between doses was performed in two stages Washing for 10 seconds, an interval of 5 seconds and then washing for 5 seconds The total volume of wash fluid was 50–60 ml from supply bottles of suitable height The bath was supplied with air

In other experiments we used an automatic assay apparatus from Casella Electronics Ltd, London The bath volume was about 3 ml, and the fluid was changed by emptying the vessel and refilling from stock bottles The washing between doses consisted in a threefold change of the wash fluid

II *Rat uterus* The procedure was as described previously (BRISEID JENSEN & SUND 1960) with some modifications The loads on uteri ranged from 0 8 to 1 3 g, depending on the agonist employed The period of contact of active solution ranged from 30 to 70 seconds The intervals between doses, in the range 3½ to 4 minutes, were fixed for each experiment The thyme was added as antagonist 1½–2 minutes before adding the agonist All the experiments were done in the automatic assay apparatus mentioned above in connection with the work on the guinea pig ileum

III *Rat duodenum* The proximal 3–4 cm segment of the duodenum of a rat weighing 150–250 g was suspended in de Jalon solution at 30° (±0 1°) Otherwise the apparatus and the procedure were as described for the guinea pig ileum

IV *Guinea pig lungs* The method of SCHILD & ARUNLAKSHANA (1959) was used

V *Rat blood pressure* Male rats weighing 250–350 g were anaesthetized with a solution containing 6% w/v of urethane and 4% w/v of sodium barbitone 1 ml/100 g was injected intraperitoneally The trachea was cannulated and artificial respiration applied Blood pressure was recorded from a carotid artery (with heparin in the polythene cannula) on a mercury manometer Thyme extracts were diluted with 0 9% sodium chloride and injected alone or together with other substances into a femoral vein

C *Materials*

Acetylcholine Acetylcholine chloride, crist, Hoffmann-La Roche & Co, A G Basel Switzerland

Adrenaline Adrenalin cryst E Merck A G Darmstadt Germany

Bradykinin Bradykinin (synthetic) in ampoules of 100 µg/ml, Sandoz, A G, Basel Switzerland

Histamine Histamine di-HCl, Light & Co, Ltd Colnbrook, England

Oxytocin Syntocinon ®, Sandoz A G, Basel, Switzerland

Serotonin 5 hydroxytryptamine creatinine sulphate, Hoffmann La Roche & Co Ltd Basel, Switzerland

The doses of acetylcholine and serotonin mentioned refer to the salts used, the adrenaline, bradykinin and histamine values are expressed as base, the doses of oxytocin are given in I U

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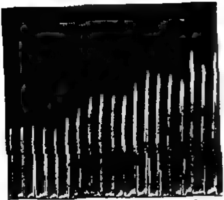

Fig 2. The effect of thyme on the acetylcholine stimulated guinea pig ileum
Gradual reduction of thyme concentration
Acetylcholine concentration 6 ng/ml Thyme (T) µg/ml Wash fluid (W) instead of thyme solution
The letters refer to the marked and the subsequent cycles

Table 1

Thyme inhibition of the isolated guinea pig ileum
Comparative experiments on the effects against different agonists giving sub-maximal contractions

Ileum preparation	Agonist	Agonist concentration ng/ml	Thyme concentration inhibiting by about 50%, µg/ml
1	Acetylcholine	15	100
	Bradykinin	20	not attainable with 50 000
2	Acetylcholine	3	50
	Histamine	3	not attainable with 10 000 excessive effect with 100 000
	Serotonin	100	2 000

B *Rat Uterus*

Contrary to what has been stated above about the guinea pig ileum, thyme occasionally stimulated the isolated rat uterus Fig 3 shows results of an experiment in which thyme at a concentration of 100 µg/ml initiated regular contractions When the thyme was then washed out and acetylcholine added instead, the latter was at first strongly inhibited Atropine did not abolish the stimulation by thyme Because of the rather irregular thyme effect, it should be mentioned that in the experiment in question bradykinin and pilocarpine had been given Pilocarpine does not

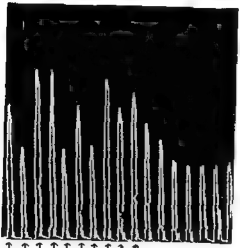

Fig 1 The effect of thyme on the acetylcholine stimulated guinea pig ileum Development of inhibition
Acetylcholine (a) concentration 10 ng/ml
– (b) – 5 ng/ml
– (c) – 2 5 ng/ml
Thyme (T) – 40 μg/ml
The letters refer to the marked and the subsequent cycles

and accordingly a higher significance of changes in sensivity, the comparisons of the lung preparations were less accurate than those of the other muscles mentioned above

4. Results

A *Guinea Pig Ileum*

The only important thyme effect recorded on the isolated guinea pig ileum was the inhibitory one, but concentrations just too low to inhibit sometimes slightly potentiated an agonist contraction Fig 1 shows an experiment in which acetylcholine was the agonist A concentration of 40 μg/ml of thyme developed in the course of 4 cycles its maximum inhibiting effect, reducing a submaximal acetylcholine contraction by about 50% Fig 2 shows, from another experiment, the effect of logarithmically decreasing thyme concentrations For the muscle to adjust itself to another antagonist concentration under the conditions chosen 2–3 cycles were necessary

Representative examples of experiments on the relative effectiveness of thyme in inhibiting various agonists are shown in table 1 Acetylcholine was strongly inhibited, 40 times higher doses of thyme were necessary for serotonin, and extremely high doses inhibited bradykinin and histamine only slightly

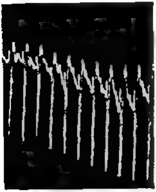

Fig 4 The effect of thyme on the bradykinin relaxed rat duodenum Increased relaxation.
Bradykinin (B) concentration 5 ng/ml Thyme (T) concentration 500 µg/ml
The letters refer to the marked and the subsequent cycles

When serotonin was used as agonist, thyme concentrations from 100 µg/ml to 2500 µg/ml, according to the sensitivity of the uterus, gave a significant potentiation of the agonist The effect was readily reversible, when thyme was washed away, the serotonin contractions settled down to the original level in the course of 2 or 3 cycles

Table 2 shows the results of comparative inhibition experiments against different agonists Apart from the observations with serotonin as agonist, the results were rather similar to those obtained with guinea pig ileum Doses of 10–100 µg/ml inhibited the acetylcholine contractions strongly, whereas concentrations up to 100 mg/ml did not significantly influence the bradykinin or the oxytocin contractions

C *Rat Duodenum*

Addition of thyme as an agonist to rat duodenum resulted in a gradual relaxation with a fall of the recorded base line The inhibiting effect of bradykinin on the muscle was slightly potentiated when thyme was added as an antagonist, and the bradykinin relaxations decreased only slowly when the thyme was washed out again Fig 4 shows the potentiation of bradykinin by 500 µg/ml of thyme The thyme effect, which had not developed to its maximum, corresponded roughly to a doubling of the bradykinin dose

When acetylcholine was used for stimulating the rat duodenum, the contraction height increased over 15–25 cycles to a stable level The increase was due to a gradual fall of the base line while the contraction peaks

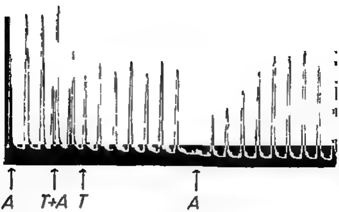

Fig 3 Irregular thyme effect on the acetylcholine stimulated rat uterus, stimulation instead of inhibition
Acetylcholine (A) concentration 150 ng/ml Thyme (T) concentration 100 µg/ml
At the third arrow thyme was withdrawn as antagonist and replaced acetylcholine as agonist. The letters refer to the marked and the subsequent cycles.

Table 2.

Thyme inhibition of the isolated rat uterus
Comparative experiments on the effects against different agonists giving submaximal contractions

Uterus preparation	Agonist	Agonist concentration ng/ml, mU/ml for oxytocin	Thyme concentration inhibiting by about 50% µg/ml
1	Acetylcholine	2 000	100
	Oxytocin	25	Not attainable with 100,000
2	Acetylcholine	50	10
	Bradykinin	0 1	Not attainable with 100 000
	Serotonin	5	Very weak inhibition with 500, Potentiation with 2,500
3	Acetylcholine	100	25
	Serotonin	20	No effect with 250 Potentiation with 2,500

normally stimulate rat uterus, but potentiates bradykinin and some other stimulating substances (BRISEID JENSEN & DYRUD, unpublished observations).

Usually the effect of thyme on the acetylcholine contractions was an inhibition, which developed and was abolished by washing again, in much the same way as was the inhibition of the guinea pig ileum The doses were also of the same order as for the ileum; the antagonist concentration inhibition curves, however, appeared considerably steeper for rat uterus than for the guinea pig ileum

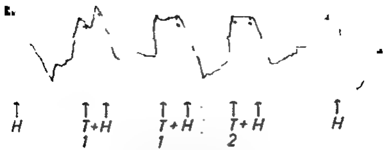

Fig 6 The effect of thyme on histamine-contracted guinea pig lungs
Unsignificant inhibition
Histamine (H) dose 1 µg Thyme (T) doses in mg

E *Rat Blood Pressure*

In doses up to at least 120 mg thyme apparently had no pronounced effect on blood pressure When it was given together with adrenaline, the rise in blood pressure was not affected by the thyme doses tested (1 to 20 mg) When small doses of thyme (1 mg) were given just before acetylcholine, the depressor effect was not altered, but the responses became more irregular than before

5. Discussion

The results obtained in experiments on thyme inhibition with different agonists on various types of isolated smooth muscles all showed that extracts of herb dried by infra red irradiation specifically inhibited acetylcholine Serotonin induced contractions were also diminished, although by considerably higher thyme concentrations, but the stimulating effect of histamine and especially those of oxytocin and bradykinin were hardly influenced

That the active principles of thyme had themselves a relaxing effect on smooth muscle was seen in experiments with rat duodenum in which the bradykinin relaxation was potentiated In experiments with guinea pig lungs the bronchi immediately relaxed to some extent when thyme was added to the unstimulated preparation

For the most thyme sensitive preparations (rat uterus and guinea pig ileum) the concentrations necessary for acetylcholine inhibition were in the range 10–100 µg/ml of the dried herb As it is hardly probable that the unknown active principle (or principles) was present in the herb in large amounts, it must be a highly active substance If, for example, a content of 1‰ is assumed for the dried herb, this means that in some preparations

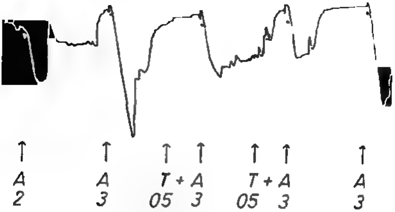

Fig 5 The effect of thyme on the acetylcholine contracted guinea pig lungs Significant inhibition
Acetylcholine (A) doses in μg Thyme (T) doses in mg

reached the same height Thyme inhibited acetylcholine and pilocarpine rather strongly, but somewhat higher concentrations were needed than for guinea pig ileum and rat uterus Serotonin-induced contractions were also inhibited by thyme, but exceptionally high concentrations of serotonin had to be used (400–800 ng/ml), and the agonist was not particularly suitable for rat duodenum stimulation

D *Guinea-pig Lungs*

When thyme was added to the perfusion fluid, a small relaxation of the bronchi was usually observed Histamine and acetylcholine were used as agonists in the experiments Fig 5 shows an experiment in which a dose of 500 μg of thyme reduced the contraction caused by 3 μg acetylcholine to about half, roughly corresponding to the contraction caused by 2 μg acetylcholine The 15-minute intervals needed between agonist doses made reliable comparisons between agonists difficult, but in the same lung preparation, as shown in fig 5, contractions initiated by 1 μg histamine were not inhibited or only slightly inhibited by 1–2 mg of thyme (fig 6) On both figures a small but distinct relaxation after addition of thyme can be seen for most of the doses added The thyme doses were given 5 minutes before the agonist doses

Bradykinin was also tested as agonist on the lung preparation, but it proved difficult to obtain reproducible contractions, as the effect subsided more or less quickly The sensitivity of different preparations to bradykinin also varied considerably, doses from about 0 1 μg to 12 μg being necessary

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of rat uterus concentrations of 10 ng/ml of the hypothetical compound nearly abolished the effect of a submaximal acetylcholine dose.

The somewhat specific inhibitory effect on acetylcholine might suggest an atropine-like action of the active principle of thyme. However, a number of experiments on serotonin-stimulated rat uteri indicated that the thyme effect must be more complicated. On uteri in which thyme extracts inhibited acetylcholine normally, the serotonin-induced contractions were potentiated. It must, nevertheless, be remembered that fairly high concentrations of thyme had to be used to obtain such an effect, and heterogeneity of the crude herb extract might well account for the observation. A further purification of the active substance or substances will be necessary before a more thorough pharmacological analysis can be carried out.

Summary

The spasmolytic properties of thyme (Thymus vulgaris L) have been examined on various isolated smooth muscles (guinea pig ileum, rat uterus, rat duodenum, guinea pig lungs) and against different agonists (acetylcholine, serotonin, histamine, bradykinin, oxytocin). Extracts prepared from herb that had been quickly dried by infra-red irradiation or from alcohol stabilised herb were considerably more active than those from herb dried at 20°–23°. The results confirm the observation of LENDLE & LU-FU-HUA (1937), who found that fresh thyme actively relaxed the pilocarpine-stimulated lungs of spinal cats, whereas dried herb usually had no such effect.

In muscle of all the types used for our experiments thyme strongly inhibited acetylcholine-initiated contractions. Serotonin-induced contractions also were diminished, although by considerably higher thyme concentrations, whereas the stimulating effects of histamine, oxytocin and bradykinin were only unspecificically affected.

In the most thyme-sensitive preparations the amounts of thyme extracts needed to reduce submaximal acetylcholine contractions by about 50% corresponded to 10–100 µg dried herb per ml. This suggests that the active principle of the herb must be highly potent.

The thyme extracts did not only inhibit substances stimulating smooth [illegible]

bradykinin was potentiated, but the stimulating effects of acetylcholine and of pilocarpine were strongly inhibited.

A further purification will be necessary before a more thorough pharmacological analysis of the active principle(s) of thyme can be performed.

the CO_2 tension in the tissues tended to rise and produced vasodilatation and respiratory stimulation (LUNDHOLM 1958) In order to verify experimentally the physiological significance of lactic acid acidosis, it would have been desirable selectively to block lactic acid production in the muscle during exercise, without influencing other physiological reactions This, however, was impracticable, because the agents that might have been used – *monoiodoacetic acid and sodium fluoride* – had too many side effects The problem had to be attacked from another angle

It is well known that during hypoxia in man, respiratory alkalosis develops as a result of the hyperpnoea, and in acclimatization to hypoxia there is a decrease in the bicarbonate concentration of the blood (for review, *vide* STICKNEY & LIERE 1953, WINTERSTEIN 1955) This has also been shown in experiments on mice (CLARK & OTIS 1952) – The question we posed was if, in acute experiments, alkalosis was prevented or changed to acidosis, would the resistance to hypoxia then be altered, and would an increase in the alkalosis influence that resistance?

In experiments on white mice, we accordingly subjected the animals to continuously increasing hypoxia and determined the lethal O_2 partial pressure, that is the O_2 tension at which respiration ceased Other groups of white mice were pretreated by subcutaneous injection of hydrochloric acid or sodium bicarbonate, after which the lethal O_2 partial pressure was determined Lactic acid was not used, being oxidized fairly rapidly by the organism, so that a constant degree of acidosis would have been impracticable In the different groups of animals the oxygen consumption was determined at various O_2 partial pressures

Methods

Male white mice weighing 15–20 grams were used Five mice were placed simultaneously in a BENEDIKT metabolic apparatus of a type described earlier (LUNDHOLM & MOHME 1949)

The respiration chamber had a capacity of two litres and was immersed in a water bath at 27° It communicated with a 500 ml spirometer The respiration chamber was divided into five compartments with wire netting to restrict the spontaneous activity of the mice The air in [redacted] was pumped through soda lime and silica gel absorption vessels Before the experiments the spirometer and the tubes running [redacted] it were filled with N_2 after which the animals were placed in the respiration chamber, which was then sealed. The mice consumed oxygen and exhaled CO_2 and H_2O, which were absorbed in the soda lime and the silica gel Owing to the fall of pressure in the system, N_2 flowed from the spirometer, so that the O_2 content of the respiration chamber gradually fell. In this way, continuously increasing hypoxia was produced, from which the mice in due course died The animals could be observed through a window in the chamber lid, and when one of them ceased to breathe the total fall in the spirometer was recorded. Th[redacted] fall was also recorded when any of the other mice died. The fall in

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From the Department of Pharmacology, University of Gothenburg, Sweden

The Effects of Acidosis and Alkalosis on Resistance of Mice to Hypoxia

By

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(Received September 10, 1962)

The lactic acid acidosis that accompanies muscular work, asphyxia, cardiac insufficiency, hyperthyreosis and shock is generally regarded merely as an indicator that the oxygen supply of the tissues is deficient As to physiological significance of the metabolic acidosis as such, the general view seems to be that it is negligible Only in muscular work is lactic acid acidosis considered to be of limited importance for respiratory stimulation and vasodilatation in the muscles SCHULTZ, MORSE & HASTINGS (1935), in experiment on unanaesthetized dogs, found that during experimental metabolic acidosis induced by ammonium chloride the performance rose *coincidently with a smaller rise in the lactic acid content of the blood* than in the controls In metabolic alkalosis produced by administration of sodium bicarbonate, the performance decreased concurrently with a rise in the lactic acid content greater than that in the controls These experiments could indicate that the organism in muscular work tries to attain a certain degree of metabolic acidosis

In McArdle's syndrome the working capacity of the skelatal muscles were much reduced There were no formation of lactic acid during moderate degrees of muscular exercise or ischemic work The skelatal muscles were lacking phosphorylase – an enzyme necessary for the conversion of glycogen to glucose-l-phosphate (MCARDLE 1951, SCHMID & MAHLER 1959)

In studies on the stimulating effect of adrenaline on lactic acid production in skeletal muscle, the view was expressed that the ability of the muscle to produce lactic acid was not only an emergency measure to cover its energy requirements during inadequate oxygen supply, but also gave rise to physiological reactions – respiratory stimulation and vasodilatation – calculated to secure a sufficient oxygen supply An increase in the lactic acid content of the blood impaired its CO_2 transporting capacity, so that

Table 1

Effects of HCl and $NaHCO_3$ on resistance to hypoxia

Group	Mean lethal O_2 tension mm Hg (vol %)	No of animals	Mean O consumption ml g min at 40°, lethal O tension
Control	40 1 ± 1 46 (5 28)	70	0 025 ± 0 0010
0 135 mg HCl g	26 5 ± 0 91 (3 49)	45	0 022 ± 0 0028
0 27 mg HCl/g	25 8 ± 1 32 (3 40)	55	0 021 ± 0 0010
0 63 mg $NaHCO_3$/g	44 8 ± 1 22 (5 90)	30	0 032 ± 0 0025

Table 2

Statistical analysis of effects of HCl and NaNCO$_3$ at the mean lethal O tension

Difference between	Difference	t	P
Control – 0 135 mg HCl	13 5 ± 1 72	7 84	<0 001
Control – 0 27 mg HCl	14 3 ± 1 97	7 26	<0 001
Control – 0 63 mg $NaHCO_3$	4 7 ± 1 90	2 48	<0 02

between 14–63 mm Hg The mean lethal O_2 partial pressure was 40 1 mm Hg at 0°C and 760 mm Hg the equivalent of 5 28% v/v O_2

In the experiments on injection of hydrochloric acid the animals tolerated a far greater degree of hypoxia before respiration ceased (table 1) After 0 135 mg/g the mean lethal O_2 partial pressure was 26 5 mm Hg, with a range of 14–41 mm Hg but after 0 27 mg/g the corresponding mean was 25 8 mm Hg and the range 10–52 mm Hg The difference in tolerance with and without HCl was statistically significant (table 2)

After subcutaneous injection of 0 63 mg $NaHCO_3$, the animals' tolerance to hypoxia was somewhat decreased (table 1) The mean lethal O_2 partial pressure amounted to 44 8 mm Hg with a range of 28–53 mm Hg The difference between controls and mice treated with $NaHCO_3$ was statistically significant (table 2)

The interval between beginning the experiment and death varied with the animals' oxygen consumption and their resistance to hypoxia In the control experiments the first animals died after 115 min and the last after 158 min In the experiments with HCl the corresponding times were 145–184 min and in the experiments with $NaHCO_3$ 118–176 min The increased resistance to hypoxia on administration of HCl was thus associated with a longer duration of exposure to hypoxia

the spirometer enabled the total O_2 loss from the system to be calculated, and hence the relevant O_2 concentration as well, since the total volume of the system was known This total volume – respiration chamber, absorption vessels, pump and tubes – was determined experimentally by adding a specific amount of N_2 to the system when it was filled with air The same volume of air was released via the spirometer, which was not included in the system When the added volume of N_2 had been mixed with the air in the system by means of the pump, samples of the gas were taken and analyzed by means of the HALDANE apparatus as modified by KROGH & LILJESTRAND The principle for determining the volume of the system was thus identical with that for determining the residual volume in the lungs The total volume amounted to about 2,450 ml, but varied with the amount of gas in the absorption vessels In the individual experiments the size of the mice was also taken into account When all animals were dead, samples were also taken, in most instances for analysis of the gas in the system, in order to check the reliability of the method The difference between the experimentally observed and the calculated O_2 contents did not exceed about 0 1 per cent An important requirement, for reliability, was an unchanged CO_2 content in the system Gas analysis at the end of the experiment showed that this did not exceed 0 05 per cent, so that variations in it were negligible

Hydrochloric acid was injected subcutaneously 10–15 minutes before the beginning of the experiment, into some animals at a dose of 0 135 mg per g body weight (3 7 mEq/kg) and into others at a dose of 0 27 mg/g (7 4 mEq/kg) The solution injected was 0 5 N The amount of hydrochloric acid injected was far less than the lethal dose, which amounted to about 1 6 mg/g (L D 50) In the experiments with $NaHCO_3$, 0 [illegible] mg/g (7 5 mEq/kg) was injected

Results

Figure 1 shows the number of animals that died at the oxygen tension indicated, with a group range of 5 mm Hg The lethal O_2 tension variated

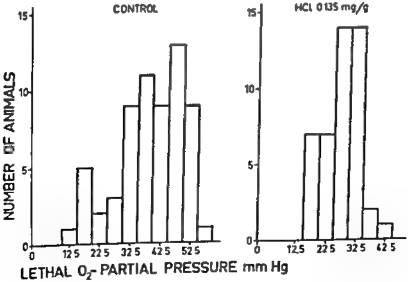

Fig 1 Frequency curves for lethal O_2 partial pressure in inspired air and the effect of 0 135 mg HCl per g

The relation between oxygen consumption and O_2 tension is evident from fig 3 The oxygen consumption, as will be seen, already began to drop at about 100 mm Hg and at 75 mm Hg it began to fall more rapidly, reaching a constant value between 45 and 30 mm Hg, when most of the animals died The decreased O_2 consumption, initially at least, was certainly attributable to a reduction in physical activity At about 75 mm Hg when the oxygen consumption began to fall more swiftly, the mice were not active, rather, the decreased O_2 consumption may well have been largely due to a reduction in oxidative processes as a result of the hypoxia

After administration of HCl the initial O_2 consumption was reduced to about 70 per cent of that in the controls This reduction must be partly attributed to decreased muscular activity The oxygen consumption fell, with increasing hypoxia, more slowly in the acidotic animals than in the controls At about 50 mm Hg the oxygen consumption in the acidotic mice exceeded that in the controls Thereafter it showed a far greater fall than in the controls before the animals died

In the mice treated with $NaHCO_3$ the oxygen consumption was initially parallel with that in the controls but at approximately 75 mm Hg the treated mice were able to maintain a higher O_2 consumption at the corresponding O_2 tension than the controls This notwithstanding they died at a less advanced stage of hypoxia

The relationship between O_2 consumption and O_2 partial pressure, however, is unlikely to be directly comparable in the different experimental groups since with a decreasing O_2 content, and the death of some animals, selection occurred in that the most resistant ones still lived These may well have been able to maintain a relatively high oxygen consumption despite a reduced O_2 content A more adequate comparison is, therefore that of the O_2 consumption of the still living animals when 50 per cent of the mice had died It has been calculated and is shown in table 1 — The table shows that the animals treated with $NaHCO_3$ had a higher O_2 consumption at 50% lethal O_2 tension than the controls The difference ($0\,007 \pm 0\,0027$ ml/g/min) was statistically significant ($P < 0\,05$) The animals treated with 0 27 mg HCl/g had, on the other hand a lower O_2 consumption at 50% lethal O_2 tension than the controls The difference ($0\,004 \pm 0\,0014$ ml/g/min) was highly significant ($P < 0\,01$)

Discussion

It is evident from the experiments described that the tolerance of mice

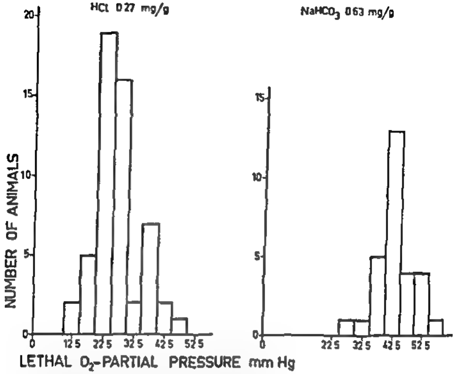

Fig 2. Frequency curves for lethal O_2 partial pressure in the inspired air and the effects of 0 27 mg HCl per g and 0 63 mg $NaHCO_3$ per g

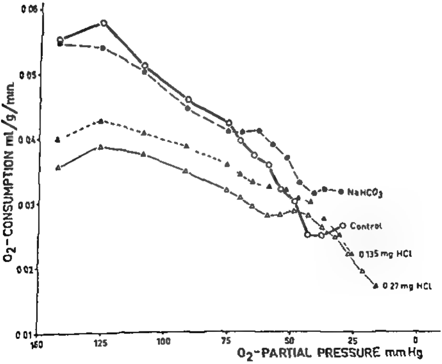

Fig 3 Oxygen consumption in mice at falling O_2 partial pressure and the effects of HCl and $NaHCO_3$.

It is well known that the alkalosis and reduced CO_2 tension in the blood accompanying hyperventilation are associated with a decreased blood flow in the central nervous system, reduced vasomotor tone and sometimes a fall in blood pressure (for review, *vide* BROWN 1953) Conversely, acidosis and elevated CO_2 tension in the blood flow to the central nervous system increase the cerebral circulation and stimulate vasomotor tone (for review, *vide* GEIGER 1958) LENNOX *et al* (1944) in experiments on human subjects, found that 3–5% CO_2 in the inspired air increased cerebral blood flow during hypoxia, amplified the electro-encephalographic activity, and delayed loss of consciousness

With an increase in the amount of fixed acids in the blood, its CO_2 transporting capacity will be impaired, for the CO_2 tension of venous blood and tissues will be higher with an unchanged blood flow and arterial CO_2 tension This is due to the fact that the curve for the relation between the CO_2 content and CO_2 tension of the blood flattens in metabolic acidosis In alkalosis due to an increase in the blood content of fixed bases, the curve, conversely, will be steeper than before (*Vide* LUNDHOLM 1958)

The significance of this change in the CO_2 transporting capacity of the blood is that, at the same CO_2 tension in the alveolar air, the CO_2 tension in the central nervous system was probably higher in the HCl treated animals than in the controls whereas in the mice treated with $NaHCO_3$ it was probably lower In view of these considerations about the influence of these considerations about the influence of CO_2 on cerebral circulation, it seems probable that the blood flow was directed to the central nervous system to a greater extent in the HCl treated animals than in the controls, whereas the reverse was probably true of the mice treated with $NaHCO_3$

Further experiments are needed to elucidate how physiological mechanisms in metabolic acidosis and alkalosis affect the resistance of mice to hypoxia The experiments, however, indicate that metabolic acidosis may be of physiological importance in hypoxia

Summary

Mice were subjected to acute progressive hypoxia and the lethal O_2 tension was determined At the same time the oxygen consumption was recorded at different partial pressures of O_2 Subcutaneous injection of 0 135–0 27 mg HCl/g substantially increased the animals' resistance to hypoxia in acute experiments, concurrently they tolerated a greater reduction in their oxygen consumption than did control animals Administration of 0 63 mg $NaHCO_3$/g reduced the resistance to hypoxia These animals had a higher oxygen consumption than the controls at a correspondingly low O_2 partial pressures

effects cannot be answered with any assurance The oxygen consumption of these animals under various experimental conditions may nevertheless serve as a basis for discussing the probable mechanism

The increased tolerance to hypoxia after acidosis was not accompanied by an augmented oxygen consumption at the mean lethal O_2 partial pressure On the contrary, these animals tolerated before respiratory arrest a greater inhibition of oxygen consumption than did the controls In metabolic alkalosis, however, respiratory arrest occurred at a higher oxygen consumption level than in the controls These observations suggest that the mechanism underlying the effects is not to be sought in an enhanced capacity of the animals to utilize the reduced amount of O_2 in the inspired air Thus, the increased tolerance to hypoxia accompanying metabolic acidosis can scarcely be attributed to compensatory physiological mechanisms, such as augmented ventilation or an improvement in the oxygen-binding or oxygen-transporting capacity of haemoglobin through a fallen body temperature or pH

The ability of the respiratory centre to function at a lower total oxygen consumption in acidotic than in control animals should therefore perhaps be ascribed to the organism's capacity for utilizing the available oxygen more "economically"

It has been amply demonstrated that a fall in body temperature parallels the decreasing oxygen consumption in animals subjected to hypoxia (LINTZEL 1931, GELLHORN 1937; BLOOD *et al* 1946) When the temperature fall was blocked, the animals showed reduced tolerance to hypoxia, whereas 3% CO_2 in the inspired air intensified the temperature depression (GELLHORN 1937) A possible explanation is that the temperature reduction was greater in the acidotic animals than in the controls, in which event the oxygen requirements of the respiratory centre may be assumed to have decreased The initially depressed oxygen consumption may favour this explanation In the acidotic animals the tissue CO_2 tension presumably increased by means of a mechanism to be discussed below After an O_2 tension of approximately 50 mm, however, the acidotic animals had a somewhat greater oxygen consumption – i e, heat production – than did the controls The demonstration of a close correlation between the depressions of body temperature and of oxygen consumption (LINTZEL 1931) argues in some degree against the assumption that the temperature reduction was greater in the acidotic animals in this O_2 tension range

Another conceivable explanation of the observed effects is that the metabolic acidosis acted on the circulation in such a way that a greater proportion of the blood flow and of the available oxygen supply was channelled to the brain and the respiratory centre

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From the Biochemical Laboratory, Northern General Hospital, Edinburgh Scotland

Detection and Determination of Hypotensive Drugs in Human Serum

By

S. L. Tompsett

(Received August 27 1962)

The detection and determination of some hypotensive drugs (hexamethonium, darenthin ®*), guanethidin) in serum has proved troublesome This is due to a lack of specific colorimetric reactions and difficulties in effecting concentration

Although there is an extensive literature on the use of hypotensive agents (c f WIEN 1961), there is a lack of data about serum concentrations

We (TOMPSETT *et al* 1961) have described a procedure for detecting and approximately determining these substances in urine A modification of the procedure has been found applicable to their examination in blood serum

Procedure

The hypotensive agents were absorbed on a column of Dowex 50 × 12 from a trichloroacetic acid extract of blood serum Elution was carried out with 5N hydrochloric acid Identification and approximate determination were carried out by paper chromatography

Preparation of Column

The column containing 3 g of Dowex 50 × 12 mesh size 200/400 – (height – 70 mm, diameter – 10 mm) was prepared as described in previous communications (TOMPSETT 1959, 1960)

Initial Treatment

To 10 ml of serum were added 30 ml of water and then 10 ml of 20% trichloroacetic acid (w/v) The mixture was centrifuged and the residue extracted twice with 20 ml quantities of 4% (w/v) trichloroacetic acid

*) Darenthin ® = bretylium tosylate (WHO NFN, BAN)

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The combined trichloroacetic acid extracts were applied to a column The column was washed with 100 ml of N hydrochloric acid, and elution was then carried out with 160 ml of 5N hydrochloric acid The acid eluate was evaporated to dryness in an all-glass vacuum still The residue was dissolved in 0 5 ml or 1 ml of water

Paper Chromatography and Detection

This was carried out essentially as described in a previous communication (TOMPSETT *et al* 1961)

100 µl (in quantities of 20 at a time), were applied to a sheet of Whatman No 1 filter paper, and development was carried out with N-butanol/acetic acid/water mixture Detection was carried out by means of the Dragendorff reagent

The R_F of any 'spots' were noted, and determinations were made by comparison with a series of standards (10, 20, 40, 60 and 80 µg) developed and detected at the same time

Standards

To 10 ml quantities of serum 100, 200, 400, 600 and 800 µg of hypotensive drug were added The complete procedure was carried out

The final extracts may be preserved for a number of separate examinations

The recovery of hypotensive drugs from column eluates was found to be within the range 78 6 to 83 1% For this purpose the eluates obtained from a number of columns were combined, and determinations were made by the colorimetric reineckate technique (TOMPSETT *et al* 1961)

Discussion

Quantities of 10, 20, 40, 60 or 80 µg of hexamethonium, bretylium or guanethidin may be readily differentiated on paper chromatograms after spraying with the Dragendorff reagent The Sakaguchi reaction may be applied to the detection and approximate determination of guanethidin within the same range

The technique as described allows determinations in serum down to 10 µg/ml, but determinations of even lower levels are possible

Summary

A procedure has been described for the detection and approximate determination of some hypotensive agents in serum

Preparation of Column

The column containing 30 g of Dowex 50 × 8 – mesh size 200 400 – (height – 140 mm diameter – 15 mm) was prepared as described in a previous communication (TOMPSETT 1961)

Initial Treatment

A 100 ml quantity of urine (+1 ml of 10 N hydrochloric acid) was applied to the column The column was washed with 200 ml of 0 1 N hydrochloric acid and then with 400 ml of water Elution was carried out with 300 ml of 33% (6 N) aqueous ammonia solution, basic substances containing a free phenolic or carboxylic acid group only being removed (TOMPSETT 1961) The ammoniacal solution was evaporated to dryness in an all glass vacuum still The residue was dissolved in a small volume of water

Paper Chromatography and Detection

Of the solution 20 µl were applied to a sheet of Whatman No 1 filter paper, and development was carried out with n-butanol acetic acid/water (4/1/5) – organic phase Detection was carried out by means of the Dragendorff reagent

The R_F of any 'spots' were noted, and the determination was made by comparisons against a series of standards (50, 100, 200 and 400 µg) developed and detected at the same time

The lower limit of detection was 50 µg, but there was ready differentiation between the standards Solutions of the standards were prepared in exactly the same way as those of the unknowns

The orange red spots did not develop immediately but only after the paper had dried

Dried residues of the eluates were also examined by the esterification technique of PLOTNIKOFF *et al* (1952) These were refluxed with a mixture of 2 ml of concentrated sulphuric acid and 20 ml of ethanol for 2 hours The mixture was then diluted to 50 ml with water The 'pethidine' content was determined by the indicator method of EL DARAWAY & TOMPSETT (1956) Recoveries of 2, 4 and 8 mg of pethidinic acid ranged from 49 2 to 54 1% of the original As a further aid to identification of the esterified pethidinic acid, the product was examined by paper chromatography after its extraction by an organic solvent

Results and Discussion

In the n butanol/acetic acid/water system, pethidinic acid has an R_F of 0 64

The immediate appearance of an orange spot when paper chromatograms are treated with the Dragendorff reagent indicates a compound that does not possess a free carboxyl group, whereas compounds having a free carboxyl group give the reaction only after the paper has dried The latter compounds are also relatively insensitive towards the reagent

Normal human urine contains an unidentified substance, found in

Acta pharmacol et toxicol 1962, 19, 368–370

From the Biochemical Laboratory, Northern General Hospital,
Edinburgh, Scotland

The Detection and Determination of Pethidinic Acid in Urine

By

S L. Tompsett

(Received August 27, 1962)

Pethidine (meperidine (USAN), demerol ®) is one of the recognised drugs of addiction and in Great Britain is the most important after morphine (DRUG ADDICTION 1961)

The metabolism of pethidine has been studied in the human and other species (MCDONALD *et al* 1946, PLOTNIKOFF *et al* 1952, 1956, BURNS *et al* 1955), and existing knowledge has been recently summarised (WILLIAMS 1959, STEWART & STOLMAN 1960) It would appear that little unchanged pethidine is to be found in urine, the main excretory products being those due to de-esterification and de-methylation In man, the main excretory products have been shown to be pethidinic and nor-pethidinic acids, the former apparently predominating There is apparently little difference in the metabolism of pethidine between normal subjects and addicts

Scanty information is available about the identification and determination of these metabolites The procedure described below permits the detection and approximate determination of pethidinic acid in urine

Pethidinic acid is absorbed from urine on a cation exchange resin The substance is then eluted with ammonia Identification and approximate determination is then carried out by means of paper chromatography and the use of the Dragendorff reagent

Procedure

The pethidinic acid used was prepared by the alkaline hydrolysis of pethidine

Preparation of Column

The column containing 30 g of Dowex 50 × 8 – mesh size 200 400 – (height ~ 140 mm diameter ~ 15 mm) was prepared as described in a previous communication (TOMPSETT 1961)

Initial Treatment

A 100 ml qua [illegible]
column The col [illegible]
with 400 ml of [illegible]
ammonia soluti [illegible]
group only being removed (TOMPSETT 1961) The ammoniacal solution was evaporated to dryness in an all glass vacuum still The residue was dissolved in a small volume of water

Paper Chromatography and Detection

Of the solution 20 µl were applied to a sheet of Whatman No 1 filter paper, and development was carried out with n butanol acetic acid water (4/1/5) – organic phase Detection was carried out by means of the Dragendorff reagent

The R_F of any 'spots' were noted and the determination was made by comparisons against a series of standards (50 100 200 and 400 µg) developed and detected at the same time

The lower limit of detection was 50 µg but there was ready differentiation between the standards Solutions of the standards were prepared in exactly the same way as those of the unknowns

The orange red spots did not develop immediately but only after the paper had dried

Dried residues of the eluates were also examined by the esterification technique of PLOTNIKOFF *et al* (1952) These were refluxed with a mixture of 2 ml of concentrated sulphuric acid and 20 ml of ethanol for 2 hours The mixture was then diluted to 50 ml with water The 'pethidine' content was determined by the indicator method of EL DARAWAY & TOMPSETT (1956) Recoveries of 2 4 and 8 mg of pethidinic acid ranged from 49 2 to 54 1% of the original As a further aid to identification of the esterified pethidinic acid the product was examined by paper chromatography after its extraction by an organic solvent

Results and Discussion

In the n butanol/acetic acid/water system, pethidinic acid has an R_F of 0 64

The immediate appearance of an orange spot when paper chromatograms are treated with the Dragendorff reagent indicates a compound that does not possess a free carboxyl group, whereas compounds having a free carboxyl group give the reaction only after the paper has dried The latter compounds are also relatively insensitive towards the reagent

an unidentified substance, found in

extracts prepared as described above and appearing on paper chromato grams at R_F 0 51 after treatment with the Dragendorff reagent No other spots have been noted

Pethidinic acid produces an insoluble reineckate in aqueous solution at *pH* 1 2 This is soluble in acetone and could form the basis of a non specific colorimetric method for the determination (TOMPSETT *et al* 1961) This reineckate does not dissociate on paper chromatograms when development is carried out with the n butanol/acetic acid/water system An insoluble reineckate is not produced in aqueous solution at neutral reactions

Ecgonine, obtained by the de esterification of cocaine, could possibly be present in the urinary extracts described above This substance does not produce an insoluble reineckate in neutral or acid aqueous solutions and is not detectabe in neutral or acid aqueous solutions and is not detectable on paper chromatograms after treatment with the Dragendorff reagent

The other metabolite of pethidine – nor pethidinic acid – was not examined, since none was available

Summary

A method is described for the identification and approximate determina tion of pethidinic acid a major metabolite of pethidine in man

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Acta pharmacol et toxicol 1962, **19**, 371–376

From the Department of Pharmacology University of Goteborg,
Goteborg Sweden

Comparison between Effects of Carinamide and Probenecid on Thiosulphate Secretion in the Renal Tubules of the Dog

By

Fredrik Berglund

(Received September 10 1962)

Thiosulphate is secreted by the renal tubules of *Lophius* (BERGLUND & FORSTER 1958) the cat (EGGLETON & HABIB 1949) and the male dog (FOULKS *et al* 1952) It is both secreted and reabsorbed in man (BUCHT 1949) and in the female dog (FOULKS *et al* 1952) The secretion is inhibited by carinamide[1]) but not by probenecid[1]) (= benemid ®) (BUCHT 1949, FOULKS *et al* 1952, BERGLUND & FORSTER 1958) The different effects of carinamide and probenecid on thiosulphate transport are somewhat remarkable since the two drugs have identical inhibitory effects on the tubular secretion of penicillin (BEYER *et* al 1951) BERGLUND (1960) investigated the secretion of the two drugs in the aglomerular kidneys of *Lophius* Carinamide reached 10–20 times as high concentrations in the urine as in plasma whereas probenecid levels in the urine were lower than in plasma It was concluded that carinamide was actively secreted by the tubules and during the transport through the cells inhibited the secretion of thiosulphate whereas probenecid was not secreted and thus did not affect thiosulphate

WEINER WASHINGTON & MUDGE (1960) showed, however, in the dog that probenecid was excreted when the urine was alkaline and that the excretion was depressed by PAH (p aminohippurate) They concluded that probenecid was secreted actively and reabsorbed by a *non ionic* diffusion mechanism This would render invalid the theory of Berglund

It still seemed possible to explain the lack of effect of probenecid in *Lophius* and in the non alkalotic dog by the absence of probenecid from the acid urine The effect of ...

[1]) Carinam de is p-(benzyl ...
Probenecid is p-(d ...
S nce the molecula ...

dog and compared with the effect of carinamide at equivalent excretion rates The results derive partly from earlier work (dog K, BERGLUND, HELANDER & HOWE 1960), partly from new experiments (dog N)

Methods

Clearance experiments were performed on female mongrel dogs in the standing position by the technique described earlier (BERGLUND, HELANDER & HOWE 1960) Creatinine was determined by the alkaline picrate method of BONSNES & TAUSSKY (1945) Thiosulphate was determined on tungstic acid filtrates of plasma by the iodate method and directly on urine by the iodine method of GILMAN, PHILIPS & KOELLE (1946) Carinamide was determined by the method of BRODIE, LEVY & BERNSTEIN (1957) and probenecid by the spectrophotometric method of TILLSON *et al* (1954)

Results

Under the experimental conditions recorded in table 1, probenecid exerted no effect on the tubular transport of thiosulphate In the three parts of the experiments, there was an average net secretion of thiosulphate of 25, 24 and 25 μmoles/minute, in that order The plasma level of probenecid in the last two thirds of the experiment averaged 153 and 194 μg/ml, with urinary excretion rates of 0 46 and 2 31 mg/min The rise in urinary excretion of probenecid was produced by administering sodium bicarbonate, with a concomitant rise in urine pH from 6 to 8

These results should be compared with the effects produced by carinamide Earlier experiments (BERGLUND, HELANDER & HOWE 1960) showed that almost maximal inhibition of thiosulphate secretion was reached at carinamide levels of 20 μg/ml plasma (dog K, fig 1) Experiments on Dog N showed fair agreement with earlier results (fig 1)

The effect of varying the urinary excretion of carinamide, by means of sodium bicarbonate, was shown in exp 131 (table 1) In the control

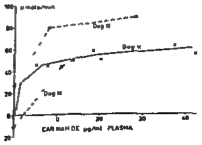


Fig 1 ... transport in two dogs x- ... -o *Dog N, 22 kg*

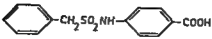
Table 1

Comparison between probenecid and carinamide at different plasma levels and excretion rates Dog N, 22 kg V — Urine flow GFR = Glomerular filtration rate

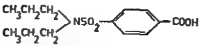
Time min	V ml/min	GFR ml/min	Thiosulphate Plasma μmole/ml	Thiosulphate Urine μmole/min	Thiosulphate Re-absorbed μmole/min	Drug Plasma μg/ml	Drug Urine mg/min
	Exp 119						*Probenecid*
− 65	400 ml H_2O orally						
0	Prime 2 3 g creatinine 1 5 g $Na_2S_2O_3$ 5 H_2O						
	Infusion 1 2% creatinine 5% $Na_2S_2O_3$ 5 H_2O 1 4 ml/min						
18 26	2 0	87	1 09	131	−36		
26– 34	2 0	82	1 32	131	−23		
34– 42	1 5	72	1 44	120	−17		
45	[illegible]						
60– 68							0 43
68– 76							0 49
76– 82							0 46
[illegible]	Prime 70 ml 5% $NaHCO_3$						
89	Infusion 3 as nr 1 + 0 8% probenecid + 5% $NaHCO_3$						
108–116	3 4	84	1 56	159	−28	193	2 30
116–124	2.9	81	1 59	154	−25	194	2 33
124–132	3 0	85	1 52	151	23	196	2 31
	Exp 131						*Carinamide*
− 15	400 ml H_2O orally						
[illegible]	Prime 2 g creatinine 2 2 g $Na_2S_2O_3$ 5 H_2O						
	Infusion 1 2% creatinine 4 5% $Na_2S_2O_3$ 5 H_2O 1 4 ml min						
25– 33	1 6	99	1 75	177	− 4		
33 41	1 6	98	1 70	181	−15		
41 49	1 6	100	1 68	182	15		
51	[illegible] 5 H_2O, 0 35% carina[illegible]						
66– 74					49		1 81
74– 82	1 4	100	1 73	130	43	13	2 04
82 90	1 3	100	1 73	115	58		1 96
90– 93	Prime 60 ml 5% $NaHCO_3$						
93	Infusion 3 as nr 2 + 5% $NaHCO_3$						
108–116	2 1	100	1 73	127	46		3 16
116–124	2 1	97	1 73	122	45	11	3 28
124–132	3 0	100	1 78	136	42		3 75

periods there was a net secretion of thiosulphate averaging 11 μmoles/min With carinamide there was net reabsorption of thiosulphate, averaging 50 and 44 μmoles/min The simultaneous plasma levels of carinamide averaged 13 and 11 μg/ml with excretion rates of 1 9 and 3 4 mg/min

Thus at suboptimal plasma levels of carinamide (< 20 μg/ml) a marked rise in its urinary excretion rate did not enhance its effect on thiosulphate secretion



CARINAMIDE



PROBENECID

Fig 2 Structural formulae of carinamide and probenecid

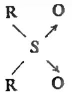
Discussion

The purpose of the study was to test the effect of probenecid on thio sulphate transport under conditions favouring the urinary excretion of probenecid By administering sodium bicarbonate the probenecid/creatinine clearance ratio was increased from 0 04 to 0 14 WEINER *et al* reported clearance ratios above 1 0, these higher ratios were evidently due to a higher dose of sodium bicarbonate (5 times ours) and higher urine flows (> 10% of GFR, compared with 4% in exp 119) Broadly our excretion rates of probenecid agree with those of WEINER *et al*

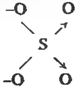
Thiosulphate transport was not affected by probenecid, in spite of plasma levels exceeding 150 µg/ml and urinary excretion rates of 2 3 mg/min Carinamide, on the other hand, already exerted maximal effect on thiosulphate secretion at a plasma level of 20 µg/ml At suboptimal levels of carinamide (11–13 µg/ml plasma) an increase in its excretion rate from 1 9 to 3 4 mg/min did not alter quantitatively the effect on thiosulphate transport

The following conclusions may be drawn 1 The inhibition of thiosulphate secretion should be correlated with the plasma level of carinamide and not with its concentration in the urine 2 The absence of effect of probenecid is probably not due to its low concentration in the urine 3 The difference in effect between carinamide and probenecid on thio sulphate secretion seems to be due to some specific difference in structural configuration (fig 2)

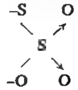
The position of the sulfone group might be the significant factor The structural similarity between sulfone, sulfate and thiosulfate is indicated by the formulae, which may be written



Sulphone



Sulphate



Thiosulphate

Arrows indicate »semipolar bonds« The sulfur oxygen bond distance averages 1 45 Å in a number of sulfones and sulfate ions (Price & Oae 1962) and 1 48 Å in sodium thiosulfate (Taylor & Beevers 1952)

In probenecid, the sulfone group is directly attached to a benzene ring probably causing strong interaction between these groups (Price & Oae 1962) – In carinamide, the sulfone group is not directly bound to any unsaturated group and its properties might be more similar to those of sulfate and thiosulfate Carinamide might therefore show affinity to a sulfate thiosulfate carrier in the tubule cells and thus inhibit the secretion of these ions

Summary

The effects of carinamide and probenecid on thiosulphate secretion by the renal tubules were studied in the dog Carinamide clearly inhibited secretion at plasma levels of 11–13 µg/ml with simultaneous urinary excretion rates of 1 9–3 4 mg/min Probenecid exerted no effect even at plasma levels above 150 µg/ml, and urinary excretion rates of 0 5–2 3 mg/min The difference in effect of the two drugs on thiosulphate secretion is probably not due to different urinary excretion rates It is suggested that the SO_2⁼ group in carinamide shows affinity for a thiosulphate-carrier in the tubule cells The SO_2⁼ group in probenecid may have lost this characteristic owing to direct attachment to a benzene ring

Acknowledgement

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Carinamide was generously supplied by the Merck, Sharp and Dohme Research Laboratories by courtesy of Dr John Baer Probenecid was generously supplied by A B Astra Sodertalje Sweden

Skilful technical assistance was given by Mrs Mona Agren and Miss Monica Johansson

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From the Research Institute of National Defence, Dept 1, Sundbyberg 4, Sweden

Thialisobumal* (Baytinal ®) as an Intravenous Anaesthetic for Rabbits

By

Bo E:son Schildt and Evert E:son Schildt

(Received October 5, 1962)

In research with small animals there is need for an anaesthetic suitable for short surgical procedures The ideal anaesthetic would be one that produces a short and sufficiently deep anaesthesia with a rapid recovery Although some fulfil the first part of this requirement, many current anaesthetics do not completely satisfy the second Many anaesthetics leave the animal in a post anaesthetic non-physiological condition that interferes primarily with respiration and circulation This means that no function can be considered normal for various periods afterwards

The requirement for a short-acting anaesthetic has arisen from a planned study on rabbits with combined injuries, that is, injuries from ionizing radiation combined with thermal or mechanical injury or both Good clinical experience with baytinal ® (thialisobumal sodium) in man gave a further impetus to this investigation

Baytinal ® is a thiobarbiturate (the sodium salt of 5,5-allyl-2-methyl-propyl thiobarbituric acid) It is usually used as a 10% solution for injection and its action in man is transient and characterized as "ultra-short" Information about its time of action in animals varies No published reports can be found about the use of baytinal ® in rabbits, but in Germany some investigations have been made on other species The first report of baytinal ® used on cats was published by AHLERS in 1957 Teuscher has reported the use of it on swine in 1958 In 1959 TEICHMANN used the anaesthetic on both dogs and cats He found that 2 cats out of 3 died from fatty degeneration of the liver, a condition which FREY earlier (1957) had attributed to the lack of liver glycogen PAUFLER (1960) studied the value of premedication before anaesthesia produced with baytinal ® in dogs

* Buthalital (WHO) = Buthalitone (BAN) as sodium salts

Baytinal ® Farbenfabriken Bayer AG Leverkusen, has been placed at our disposal by AB Delgar, Stockholm

Thiobarbiturates in general are characterized by a short anaesthetic but a fairly long post anaesthetic stage This is partly explained by the fact that BAYTINAL, at any rate, is rapidly eliminated from the blood and taken up by lipoid containing cells, particularly those in the central nervous system, but also by other fat containing substances (BOLZ 1961) By this mechanism most of the anaesthetic accumulates where it cannot act specifically It has been shown in heart, lung liver preparations that decomposition of baytinal ® occurs in the liver (Merkblatt den Fabriken Bayer) The rate of decomposition is slow, and the intermediary products are hypnotic in their action According to BRODIE *et al* (1952) only 50 per cent of the drug has been eliminated at the time of recovery from ana esthesia which means that the post anaesthetic stage must be relatively long Except for its short duration of action baytinal ® has all the characteristic properties of barbiturates It is available as a yellow powder soluble in distilled water to give a basic reaction The carbon dioxide of the air alters its pH and hence its solubility Intentional perivenous injections have shown that it is not very toxic to the tissues

The action of baytinal ® on respiration and circulation is considered to be about the same as that of enhexymal sodium (hexobarbitone (B P), evipan ®) The risk of liver damage and certain toxic conditions have been considered as contra indications for repeated use of baytinal ®

General Method

Since baytinal ® can be characterized as an ultra short intravenous barbiturate, the total dose of the drug is of no interest unless the time of injection is considered Our investigation has been split into three parts

a) determination of minimum anaesthetic dose and minimum lethal dose at different rates of injection Some tests with an antagonist of barbiturates are also reported,
b) the use of baytinal ® as an anaesthetic for surgical procedures,
c) comparison of baytinal ® with mebumal sodium (pentobarbitone sodium B P)

The general method was the same in all three parts of the investigation except where otherwise stated

The classical scheme for stages of anaesthesia which was originally given by [illegible] (See table 1)

[illegible] h of anaesthesia [illegible] hods was there fore to find a mean of determining the dep[illegible] xes that give in anaesthesia a precise and reproducible response and are well correlated with the depth of anaesthesia will be the best criteria of the stage of anaesthesia The most relevant

Table I

Division of anaesthesia into four stages, according to Guedel s scheme

Stage	Name of stage
I	Analgesia
II	Delirium
III	Surgical Level 1 – 2 – 3 – 4
IV	Respiratory paralysis

reflexes are those in stage III, level 3 (see table 1), which is also called surgical anaesthesia.

Pilot tests were carried out to select reflexes that fulfil the above-mentioned requirements The reflexes selected after the preliminary studies were a) the corneal reflex, b) the Achilles tendon reflex, c) the pedal reflex

a) MORCH (1947) used a method for determining the corneal reflex with a horse hair, in which the cross-section of the hair was considered to be a function of its capacity to exert pressure on the eyeball but this method was found to be too delicate during the preliminary studies Another reason for not using it was that narcosis produced by barbiturates does not permit a clear-cut scheme of anaesthesia in the same way as does that induced by an inhalation anaesthetic Further, stage III, level 3, is not reached until the corneal reflex disappears Some fibres of cellulose were selected as an irritant for the cornea The cornea was kept moist by physiological saline, and the reflex response was graded according to the scheme – abolished reflex, +very slow reflex, ++ slow reflex, +++ normal reflex

b) The reflex for deep pain was tested by pinching the Achilles' tendon This reflex was selected because it proved to be sufficiently sensitive and disappeared earlier than the corneal reflex but later than the pedal reflex

c) The pedal reflex selected as a reflex for superficial pain was evoked by pricking the foot with a pin Disappearance of the reflex was correlated with other signs indicating that the animal was in stage III

Since the reflexes were tested by the same person as exactly as possible, sufficient precision was considered to be established

The criteria used for the smallest anaesthetic and the smallest lethal dose were as set out below

The smallest anaesthetic dose was the one that in a group of animals produced abolition of the reflex for deep and superficial pain as well as a very slow corneal reflex and the smallest lethal dose was the one that killed all the animals in a group despite artificial respiration

Baytinal ® was used as a fresh 10% (w/v) solution, which was injected into a marginal ear vein When anaesthesia had been induced, the animal was moved to an observation bench Infrared radiation and repeated turning of the animal from one

side to the other were applied to prevent post anaesthetic complications A record of anaesthetic results was set up for each animal During the post anaesthetic period it was recorded when the animals began to move their heads, turned over in the prone position, and tried to walk

Determination of Minimum Anaesthetic Dose and Minimum Lethal Dose at Different Rates of Injection

This part of the study contains determination of

a) the relation between time of injection versus anaesthetic and lethal dose,
b) the range of anaesthesia (difference between anaesthetic and lethal dose),
c) anaesthetic coefficient (ratio between anaesthetic and lethal dose)

Material

For this part of the investigation 95 rabbits were used They were of various breeds, males and females, and their mean weight was 2 18 ± 0 29 kg Many of the animals were used repeatedly but always at longer intervals than 1 week

Method

The general method has already been described In this part of the investigation, groups of 5 animals were given doses increasing from a subanaesthetic up to a lethal level at different rates of injection The general outline of this part of the investigation is shown in table 2

Different times of injection (½ 2 4 and 8 minutes) were used so as to correspond to the short duration of action of Baytinal The injection was made as continuously as possible

Table 2

Working plan for determining minimum anaesthetic and lethal doses

	Dose mg/kg						
Injection time min	50	70	90	110	130	150	170
½	5	5	5	5	5	–	
2	5	5	5	5	5	5	–
4	–	–	5	5	5	5	–
8	–	–	–	5	5	5	5

Table 3

Results of determining minimum anaesthetic and lethal doses for baytinal ®

Injection time min	Minimum anaesthetic dose mg/kg	Minimum lethal dose mg/kg	Anaesthetic range	Anaesthetic index
½	80	100	20	1 24
2	90	150	60	1 67
4	90	160	70	1 78
8	110	170	60	1 55

Table 4

Duration of anaesthetic and post anaesthetic periods at different injection times for minimum anaesthetic dose

Injection time min	Dose mg/kg	Cessation in minutes of			Time in min between injection and attempts at walking
		Corneal reflex	Achilles reflex	Pedal reflex	
½	80	5	9	17	140
2	90	2	3	4	240
4	90	14	28	38	165
8	110	20	26	47	215

Results

The results are set out in table 3

It is evident that the smallest anaesthetic dose for baytinal ® varies between 80 and 110 mg per kg body weight In the group with an injection time of ½ minute some of the animals showed adequate anaesthesia at the level of only 70 mg/kg and all animals at 90 mg/kg Transient apnoea appeared among animals in the group of only 50 mg/kg, and 2 animals died from 90 mg/kg The smallest anaesthetic dose was 80 mg/kg, but the depth of anaesthesia varied more with the longer times of injection The range of anaesthesia was small (20 mg/kg)

Surgical anaesthesia was produced at 90 mg/kg, and the animals died at 150 mg/kg if the time of injection was increased to 2 minutes Apnoea appeared in some with a dose of only 130 mg/kg The range of anaesthesia was 60 mg/kg and the anaesthetic index 1 67

With an injection time of 4 minutes the range of anaesthesia was increased to 70 mg/kg, and the anaesthetic index was 1 78 Apnoea occurred from 110 mg/kg onwards

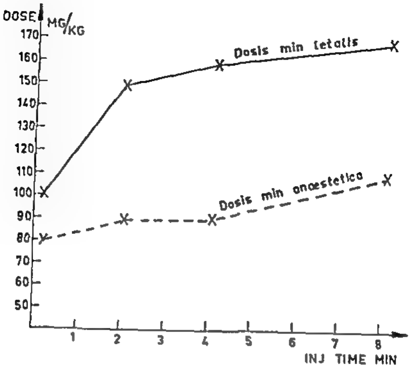

Fig 1 Dependence of injection time on anaesthetic and lethal doses of baytinal ®

If the time of injection was increased to 8 minutes, the minimum anaesthetic dose was reached at 110 mg/kg and the minimum lethal dose at 170 mg/kg The index of anaesthesia decreased to 1 55

A compilation of the results from the anaesthetic records gives the figures (means) shown in table 4

With an injection time of ¼ minute the length of the anaesthetic period will be some minutes Despite the short surgical anaesthesia, a post-anaesthetic period of about 2 hours will follow With an injection time of 4 min the length of anaesthesia will increase to 15–20 min on about the same dose and with the same length of post-anaesthetic period Further increase of injection time gives no striking advantage The length of the anaesthetic period is about the same, but that of the post anaesthetic stage will increase

The results are graphically reproduced in fig 1, which shows the dependence of minimum anaesthetic and lethal doses on the time of injection The distance between the curves, that is, the range of anaesthesia, has its highest value at an injection time of 4 min It will also be seen that the dose for anaesthesia will increase by only 30 mg/kg, but the lethal dose by a factor 2, if the time of injection varies from ¼ to 8 min

Except for apnoea, no complications occurred One animal that had

received 110 mg/kg during 3 min died 7 days later of an enteritis not directly referably to the investigation Both the induction of and the recovery from anaesthesia were smooth in all animals

Trial with an Antagonist of Barbiturates

Attempts have been made to shorten the length of the post-anaesthetic period by using different drugs that antagonize the action of barbiturates Favourable reports have appeared on bemegride especially in England (cited by Bolz)

To examine the effect of bemegride malysol ®*) 50, 100, and 200% of the dose (mg/kg) given to cats in the experiments reported above were given to a number of rabbits Injections were given between ½ hour to 4 hours after baytinal ® had been administered No significant change in the course of the post-anaesthetic stage was noticed In connection with the injection a short phase of excitement occurred

Trials concerning Addiction and Toxicity

In order to study the question of addiction and toxicity, rabbits were given baytinal ® repeatedly One animal, for instance, was given 10 doses ranging from 50 to 90 mg/kg for 1 month No change in the reaction of the an...

considered to be of the same type as those reported by Frey

Baytinal ® for Minor Surgery

Baytinal ® was tried in a number of minor surgical procedures to permit clinical evaluation and further observations of the relation between depth of anaesthesia and loss of reflexes

Material

25 rabbits (small Chinchilla) of both sexes were used for this part of the investigation None had been used earlier and all had been observed for more than a week without exhibiting any kind of disease or injury Their mean weight was 2 17 ± 0 46 kg Ten animals were given a dose of X rays (840 r) 1 hour before surgery

*) Inj malysol ® = 5% bemegride Apotekens Kompositionslaboratorium (ACO) Stockholm

Method

The general method has already been described The dose of baytinal ® va between 50 and 125 mg/kg, with a mean of 90 mg/kg One third of the calculated was injected for about 1 minute The rest of the dose was injected during abo consecutive minutes, with close observation of changes in respiration and refle Whenever depression of respiration was severe or the corneal reflex abolished or b the injection was stopped Surgery was begun within 5 min of the injection surgical procedure consisted of making an incision 2–3 cm long in the mid line of abdomen Then 10 ml of resuspended, autologous, Cr^{51} tagged erythrocytes injected by means of a rubber tube The abdomen was closed in layers, and the anir were observed in the post anaesthetic period, as described above

Results

In all animals muscular relaxation was sufficient Slight movements the legs were noticed in about half of them when the peritoneum touched Duration of surgical anaesthesia was 10–15 min The corn reflex was abolished or very slow in all animals except one, and t criterion was reached in 3 animals at the level of only 75 mg/kg Ir animals apnoea occurred after only 50 mg/kg The depth and the length anaesthesia for the irradiated animals did not show any difference fro those in the rest of the animals The post-anaesthetic stage, as measur from injection to the time when the animals began to move their hea was about 1½ hour The induction of and the recovery from anaesthe occurred without any noteworthy discomfort and no late complicatio arose

Comparison with Mebumal Sodium

Mebumal sodium*) has long been used as an intravenous anaesthet for small animals The mixture used in the present study was prepared f dogs, but was given to rabbits

Mebumal is not absorbed by fat as are the thiobarbiturates, and th allows faster excretion (STEVENSON 1958) Decomposition takes pla through a process of oxidation, which gives hypnotically active product The total time of excretion by dogs, measured as barbituric acids, between 2 and 4 days (FREY) Injected intravenously and quickly rabbits mebumal produces anaesthesia for about 10 minutes (BOLZ)

Urethane has long been used as an anaesthetic with a long duration c action and a wide narcotic range It has few toxic effects and can b administered by various routes The above mentioned mixture of urethan

*) Pentobarbital sodium (rINN)

Composition of preparation	Mebumal	1 8 g	Urethan	25 0 g
	– sodium	4 0 –	Glycerol	12 5 –
	Spir conc	15 0 –	Aq steril ad	100 ml

sthesia will allow an injection of about 75% too much baytinal ® and 50% too much mebumal There is some difference in the length of the post-anaesthetic period The animals in the baytinal ® group began to move their heads after about 1 hour and those in the mebumal group after about 3 hours If we compare the time from injection to attempts at walking, we find that the difference between the two groups has decreased to ½ hour The post-anaesthetic stage after baytinal ® can be characterized as somnolent and after mebumal as soporous

Discussion

The results show that baytinal ® has about the same action on rabbits as that reported by earlier investigators (AHLERS 1957, TEICHMANN 1959 and PAUFLER 1960) on dogs and cats Baytinal ® produces a short surgical anaesthesia and a post-anaesthetic stage of 1 hour or longer The short but adequate anaesthesia from baytinal ® is a result of its pharmacological properties Further administration of anaesthetics to maintain the depth of anaesthesia will result in another short period of anaesthesia, which will be followed by a longer and more accentuated stage of post-anaesthesia Because of this mechanism and of the risk of injuries to the liver, it is evident that baytinal ® should not be used repeatedly A certain prolongation of anaesthesia can be achieved by simultaneous administration of chlorpromazine (PAUFLER 1960) or similar drugs Anaesthesia of long duration can be produced only by changing over to another anaesthetic The pharmacological action of baytinal ® gives rise, as expected, to the same pattern of individual variation as does that of other drugs For this reason it is impossible to set up a general rule for its administration The results presented in table 2 show that the range of anaesthesia is widest with an injection time of 4 min The length of the post-anaesthetic period as shown in table 4, at this rate of injection is about the same as for 2 min, but the corneal reflex remains slow for a considerably longer period In the group with a 4 minute injection time apnoea did not occur until dosage was close to the lethal level (160 mg/kg) Hence, an injection time of 4 min is the best under the conditions of our investigation

The use of baytinal ® in the second part of this study showed that the drug can produce satisfactory surgical anaesthesia for 10 15 min Muscle relaxation was adequate, despite some movements of the legs when the peritoneum was touched The postulated correlation between loss of reflexes and depth of anaesthesia was found to be correct The reflexes for deep and superficial pain were abolished, and the corneal reflex was slow in all animals Apnoea occurred only in 2 animals that weighed considerably less than the others This was probably explained by the lack of

buffer action of fat on the effect of baytinal ® No complications were noted and baytinal ® can be regarded as a good anaesthetic for surgical procedures lasting up to 15 minutes

From the third part of the study it is evident that mebumal given in a dose of 25 mg/kg did not fulfil the criteria laid down for the smallest anaesthetic dose It can therefore be postulated that the smallest anaesthetic dose is about 30 mg/kg A lethal effect was obtained with 45 mg/kg, which thus gives an anaesthetic range of 15 mg/kg Mebumal produced anaesthesia free from complications Respiration is generally known to be affected by barbiturates, and our investigation has confirmed that baytinal ® and mebumal are no exceptions, to this rule The respiratory rate of the animals that received baytinal ® was about 50 per minute half an hour after the injection The corresponding rate for the group that received 30 mg/kg of mebumal was about 30 per minute This means that, even allowing for individual variations, the depressing effect of mebumal is probably more intense A comparison of the effects of excessive doses of baytinal ® and mebumal reveals a distinct difference Circulatory and respiratory failure as a result of overdosage of mebumal was found to be prognostically less favourable than that provoked by excessive doses of baytinal ® Baytinal ® seems to have a more selective central action on respiration, and circulation will be seriously affected only at still higher doses At higher doses of mebumal this biphasic action could not be recognized Within a short time after respiratory failure the heart stopped, and the animal died Artificial respiration after large doses of baytinal ® frequently prevented all anoxic damage until spontaneous respiration returned but did not have the same effect after mebumal

A comparison between baytinal ® and mebumal has thus shown that the anaesthetic and the post anaesthetic periods of both are about the same Baytinal ® has a wider range of anaesthesia and a less pronounced post anaesthetic stage and produces a shorter respiratory depression than does mebumal and, after overdosage the prognosis with baytinal ® is more favourable than with mebumal

Summary

Th…

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The effect of varying injection times (½, 2, 4, and 8 min) on the smallest anaesthetic dose and the smallest lethal dose was studied in 95 rabbits The optimal injection time was found to be 4 minutes, at which the results obtained were (see tables 3 and 4, and fig 1)

smallest anaesthetic dose	90 mg per kg body weight
smallest lethal dose	160 –
range of anaesthesia (difference between lethal and anaesthetic dose)	70 –
anaesthetic index (ratio between lethal and anaesthetic dose) and	1 78
duration of the post anaesthetic period	165 minutes

A short period of respiratory arrest occurred at all injection times

The practical value of baytinal ® (dose 90 mg per kg body weight) was investigated at 25 laparotomies The results were (see table 5)

> Surgical anaesthesia with sufficient muscular relaxation was produced *for at least 10 min in all cases*, induction and recovery were smooth, and there were no anaesthetic complications

A comparative study was made with 6% mebumal sodium as an intravenous agent 25 animals were used and the results obtained were (see table 5)

a *surgical anaesthesia of approximately the same length as from baytinal* ®, a post anaesthetic period of approximately the same length as from baytinal ®, a *"practical" anaesthetic range 3 times smaller than for baytinal* ®, a depressive effect on respiration for a longer period than from baytinal ®, and a wider margin between the dose producing respiratory depression and the lethal dose than that with mebumal

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From the Department of Physiology, Karolinska Institutet,
Stockholm 60, Sweden

Decrease in Plasma Potassium during the Disulfiram-Ethanol Reaction in Rabbits

By

T. Brundin, W. Green[1]) and E. S. Perman[2])

(Received September 26, 1962)

It is well known that ethanol elicits a characteristic reaction in human subjects pretreated with disulfiram (antabuse ®) (HALD, JACOBSEN & LARSEN 1948), and clinical studies have shown that hyperventilation, decreased arterial pressure, tachycardia and ECG changes are among the recognized manifestations of this reaction (HINE *et al* 1952, RABY 1956) The mechanism of the reaction has, however, to a large extent been obscure Recent work (PERMAN 1962) has shown that a similar reaction can be produced in rabbits, this extends the possibilities for experimental work on its mechanism RABY (1952) often noted a fall in plasma potassium during disulfiram-ethanol reactions in human subjects and suggested that the ECG changes during the reaction were connected with hypopotassaemia In our study plasma potassium was determined during the disulfiram-ethanol reaction in awake rabbits The haematocrit was also followed

Methods

In male rabbits (2–4 kg) the marginal vein and central artery of one ear were cannulated under local anaesthesia, and the blood pressure was recorded as described by PERMAN (1962) Samples for potassium determination were drawn through the arterial cannula before and 40 min after ethanol, and samples for haematocrit determination before and 20, 40 and 60 min after ethanol Under 8 ml blood were withdrawn in each experiment

Plasma potassium was determined by flame photometry and the conventional technique Haematocrit was determined in duplicate samples after centrifugation for 20 min at 12000 r/min in 50 mm glass capillaries

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[2]) Recipient of a grant from the Swedish State Medical Research Council

Ethanol (4 4 mmol/kg), was infused i v during 5–10 min in 4 animals not pretreated and in 6 animals pretreated with 1 + 1 g disulfiram (antabuse ®, Dumex Ltd) by stomach tube 24 and 2 hours before experiments A corresponding volume of saline was infused in 2 similary pretreated and in 2 rabbits not pretreated

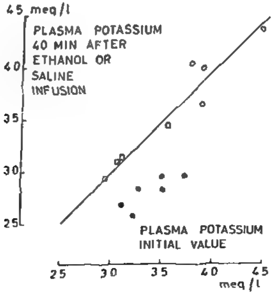
Results

Plasma potassium showed a statistically significant decrease when ethanol was administered to rabbits pretreated with disulfiram, there was also, as expected, a significant decrease in arterial pressure in this group (table 1) No overt changes in potassium (fig 1) or arterial pressure

Table 1

	No of exp	Initial values		Change 40 min after ethanol	
		Art pressure mm Hg	Plasma K+ meq/l	Art pressure mm Hg	Plasma K+ meq/l
Controls	4	93 ± 5*	3 99 ± 0 15	−3 ± 2	+0 03 ± 0 15
Pretreated with disulfiram	6	90 ± 4	3 39 ± 0 10	−23 ± 4	−0 58 ± 0 06

* Mean ± standard error of mean



... potassium content plotted against value obtained 40 min after

occurred in any of the other experiments No noteworthy changes in haematocrit were observed when ethanol was administered to rabbits pretreated with disulfiram (2 exp)

Discussion

The decrease in plasma potassium during the disulfiram-ethanol reaction in the rabbit corresponds well to what has been noted in human subjects by RABY (1952), but our experiments have yielded no information about its genesis However, PERMAN (1962) has shown an initial increase in arterial pH during the disulfiram ethanol reaction in anaesthetized rabbits, probably due to the associated hyperventilation This may be related to the potassium changes, In dogs, under certain conditions, respiratory alkalosis leads to a fall in serum potassium (SIMMONDS & AVEDON 1959 and others) – The decrease in plasma potassium during the disulfiram-ethanol reaction may be of some clinical importance Stronger indications are perhaps required for administering disulfiram to patients, such as those receiving digitalis in whom a fall in potassium might be dangerous

Summary

A significant decrease in plasma potassium occurs during the disulfiram-ethanol reaction in awake rabbits This confirms earlier results on man It is suggested that increased arterial pH, associated with the hyperventilation during the reaction, may be of importance for this hypopotassaemia

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Johan Gunnar Ahlgren

Gunnar Ahlgren died on the 1st of October 1962 For about the last 10 years he had suffered seriously from several diseases he had to undergo several major operations for repeated attacks of stomach haemorrhage In the end his not very robust nature succumbed to the burdens of his illness

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Up to about 1935 Ahlgren made numerous comprehensive scientific publications, for many years they involved the study of biological oxidation processes and micro tissue metabolism, which was then a dominating theme at the university of Lund, inspired by T Thunberg's work at the Physiological Institute True, Ahlgren's works are not characterized by great vision , they first and foremost show detailed precision and reliability As an example of Ahlgren's working methods, I should like to mention his treatise "Über die Einwirkung von Kohlensäure, Bikarbonat und H Ionenkonzentration auf überlebende Organe und ihre Beeinflussbarkeit durch Pharmaka" (Skand Arch Physiol 1930, 59, 1-24) This paper, which must be considered a classic on its subject, discusses the optimal composition of different salt solutions for different purposes For several decades we have here, at the Pharmacological Institute in Copenhagen, availed ourselves of the results of this investigation, we have often made control test and have always found Ahlgren's results exact

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became possible to establish contact with colleagues in Finland and Sweden (but not in Norway), two of them, Ahlgren and Armas Vartiainen in Helsinki, immediately joined the group of editors Not till some years later did other Scandinavian colleagues join the Editorial Board Ahlgren was always deeply interested in the growth of this journal, and the first paper to be published in the journal described work carried out at his Institute

In outward appearance Gunnar Ahlgren seemed to maintain an attitude of reserve, in order to get to know the man Ahlgren, it was necessary first to penetrate his shell of correctness and interest in formalities He was sensitive and warm hearted He would go far out of his way to help his cooperators, even though his assistance was not always properly appreciated Criticism, which no person in a prominent position can avoid, and of which he became especially a victim during recent years, he took greatly to heart

His colleagues have lost a dear friend, our journal has lost a highly valued contributor

Honoured be his name

Knud O Moller

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